



# BLOOD

*The Journal of Hematology*

---

VOLUME III, 1948



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# BLOOD

## *The Journal of Hematology*

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NOTE Of the twelve issues of BLOOD published in 1948, six were dedicated to Dr George Richards Minot as part of an Anniversary Volume in his honor. These issues, composed of papers by his colleagues, co-workers and former associates (together with a concluding issue to appear in February 1949), are to be collected in volume form early in 1949 as the George R. Minot Symposium on Hematology. The issues dedicated to Dr Minot are indicated in the following Contents by the notation (Minot Anniversary Issue).

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GEORGE R. MINOT

ANNIVERSARY VOLUME



George R. Mint

## A NOTE BY THE COMMITTEE ON THE GEORGE R MINOT ANNIVERSARY VOLUME

Following a testimonial dinner on the occasion of the sixtieth birthday of Dr George R Minot in 1945 a group of his colleagues conceived the idea of the preparation of an Anniversary Volume of manuscripts to be published in honor of Doctor Minot by his colleagues, co-workers, and former associates

A Committee was formed composed of W B Castle, Boston, W Dameshek, Boston (Chairman), R L Haden, Cleveland, E B Krumbhaar, Philadelphia, E Meulengracht, Copenhagen, Denmark, O H Pepper, Philadelphia, R M Suárez Puerto Rico, F H L Taylor, Boston (Secretary), G H Whipple, Rochester, N Y, L J Witts, Oxford, England. Invitations to contribute articles were extended to a world-wide group of active workers in the field of the blood, and to others who had in the past been closely associated with Dr Minot

It appeared eminently desirable that the Anniversary Volume should have full publication in the literature and the Publishers and Editorial Board of BLOOD, *the Journal of Hematology*, graciously opened their pages for the receipt of these manuscripts

The publication of the Anniversary Volume has been made possible by generous gifts from the Lederle Laboratories, Inc, of Pearl River, N Y, and Mr J K Lilly of Eli Lilly and Company, Indianapolis, Indiana. We especially wish to thank Mr Henry M Stratton, our publisher, for his active interest and guidance and for volunteering to underwrite personally the production of the series of articles and of the bound volumes to follow. Our thanks are also due to Dr Henry J Tagnon of the Memorial Hospital, New York, for his aid in the translation of manuscripts

The members of the 'George R Minot Anniversary Volume Committee' deem it a distinct pleasure and a great honor to dedicate these articles by his colleagues and friends to Dr George Richards Minot

### A NOTE BY THE EDITORS OF "BLOOD"

The publication of the eighty odd articles in honor of Dr George R Minot will take place in regular issues of *Blood* beginning with the January, 1948 issue, and continuing—probably in alternate issues—throughout 1948. As far as possible, the various articles will be classified and published in groups of related subjects. For example, the first issue deals primarily with pernicious anemia, and the second will deal with miscellaneous problems in anemia.

The Editors have taken the liberty of making notations and minor changes in certain manuscripts, always for the sake of greater clarity. Editorial footnotes are suitably marked, such notes represent the opinions of the Editors and not necessarily those of the authors of the papers. We apologize in advance for any errors that might have crept in, they are unintentional.

WILLIAM DAMESHEK,  
For the Editors of *Blood*

## FOREWORD

This is a foreword to the series of articles written by his friends and his co-workers to celebrate the achievements of George Richards Minot. It is a good thing thus to give recognition to high scientific accomplishment, it warms the heart of both the donor and the recipient of the recognition, and it reminds other workers that creative thinking is regarded as worthy of praise.

The history and the specific contributions of George Minot are recorded in the bibliography and curriculum vitae which appear on the following pages. Perhaps it is pertinent to inquire what qualities may be associated with this kind of accomplishment.

First of all, George Minot is George Minot, and no one in the world is quite like him. Of course, like almost everyone to whom a "Festschrift" is dedicated, he has been industrious and persistent. He had been surrounded by tradition all his life, and in some ways his reactions and his thinking are highly traditional, but this habit of mind is combined in an extraordinary linkage with an insatiable curiosity and an avidity for understanding that has driven his mind into new and startling regions of thought. That curiosity is a major motive for George Minot is attested by his own writings: "To solve human problems, an active creative imagination and scientific curiosity are necessary tools."

Indeed, the direct desire to explain something otherwise inexplicable may be said to be one of the best motives for research. Its validity in the case of George Minot is shown not only by his own classic discovery of the effectiveness of an adequate dosage of liver in pernicious anaemia, but by his capacity to stimulate curiosity in others, and to gather around himself young men who have curiosity similar to his. The articles to which this note is a foreword make up an adequate example of this side of his character.

To the desire to learn and the desire to encourage curiosity on the part of his students is to be added a great desire to be useful to the sick. If George Minot is to be judged by his motives—a zeal for knowledge, an enthusiasm for teaching, and a humane urge to alleviate suffering and disability—these motives make up a list that lesser personalities may envy.

C. SIDNEY BURWELL, Dean  
Harvard Medical School  
Boston, Massachusetts

# GEORGE RICHARDS MINOT

## BIOGRAPHICAL DATA

Born in Boston, Massachusetts, December 2, 1885 Son of James Jackson and Elizabeth (Whitney) Minot

### *Degrees*

A B cum laude	Harvard University	1908
M D cum laude	Harvard University	1912
S D (honorary)	Harvard University	1928

### *Hospital and University Appointments*

Medical House Officer	Massachusetts General Hospital	1912-13
Assistant Resident Physician	Johns Hopkins Hospital	1913-14
Assistant in Medicine and Research		
Fellow, Physiology Laboratory	Johns Hopkins Medical School	1914-15
Assistant in Chemistry	Harvard University	1915-16
Assistant in Medicine	Massachusetts General Hospital	1915-18
Assistant in Medicine	Harvard Medical School	1915-18
Visiting Physician	St. Luke's Convalescent Home	1916-18
Assistant Consulting Physician	Collis P. Huntington Memorial Hospital	1917-19
Associate in Medicine	Massachusetts General Hospital	1918-23
Physician	Collis P. Huntington Memorial Hospital	1919-23
Assistant Professor of Medicine	Harvard Medical School	1918-27
Consulting Physician	Massachusetts Charitable Eye and Ear Infirmary	1922-24
Chief of Medical Service	Collis P. Huntington Memorial Hospital	1923-28
Physician to Special Clinic	Massachusetts General Hospital	1923-25
Associate in Medicine	Peter Bent Brigham Hospital	1925-28
Special Consultant in Diseases of the Blood	Massachusetts General Hospital	1925-27
Member Board of Consultation	Massachusetts General Hospital	1927-
Clinical Professor of Medicine	Harvard Medical School	1927-28
Professor of Medicine	Harvard Medical School	1928-
Director, Thorndike Memorial Laboratory	Thorndike Memorial Laboratory	
Chief, 4th Medical Service	Boston City Hospital	1928-
Visiting Physician	Boston City Hospital	1928-30
Consulting Physician	Boston City Hospital	1928-
Consulting Physician	Peter Bent Brigham Hospital	1928-
Director, 2nd and 4th Medical Services	Beth Israel Hospital	1929-
Consultant in Hematology	Boston City Hospital	1930-32
	Palmer Memorial Hospital	
	N. E. Deaconess Hospital	1943-

### *Memberships*

Honorary Fellow Royal College of Physicians, Edinburgh	1931
Honorary Fellow Royal College of Physicians, London	1938
Honorary Fellow New York Academy of Medicine	1933
Honorary Fellow Institute of Medicine of Chicago	1933
Honorary Fellow Royal Society of Medicine, London	1932
Vice President étranger Société Française d'Hématologie	1938
Corresponding member Royal Academy of Medicine (Belgium)	1931-1939

Honorary Member Royal Academy of Medicine (Belgium)	1939
Honorary Member Kaiserlich Leopold Caroline Deutsche Akademie der Naturforscher (Halle)	1935
Honorary Member Society Biological Chemists (India)	1936
Honorary Member Finnish Society of Internal Medicine (Helsingfors)	1938
Honorary Fellow, Medical Association of Finland	1945
Fellow American Philosophical Society	1935
Fellow American College of Physicians	Prior to 1926
Fellow American Medical Association	1912
Member of Association of American Physicians	1919
Member of Council	1931
President	1938
Member of American Society for Clinical Investigation	Prior to 1920
Member of American Academy of Arts and Sciences	1927
Member of American Clinical and Climatological Association	1923
President	1932
Member of National Academy of Sciences	1937
Member of Academy of Medicine of France	1945
Phi Beta Kappa (honorary)	1929
Alpha Omega Alpha	1911
Honorary Fellow College of Physicians, Philadelphia	1947
Advisory Council of Physicians Forum	1946
President, Senior Staff, Boston City Hospital	1947

*Awards*

Kober gold medal, Association of American Physicians	1928
Charles Mickle Fellowship, University of Toronto	1928
Cameron Prize, University of Edinburgh	1930
Gold medal, National Institute of Social Sciences	1930
Gold medal and Award, Popular Science Monthly	1930
Moxon medal, Royal College of Physicians, London	1933
John Scott Medal of City of Philadelphia	1933
Gold Medal of Humane Society of Massachusetts	1935
Nobel Prize in Physiology and Medicine, jointly with William P. Murphy and George H. Whipple, for work on liver treatment of the anemias	1934
Scroll Award of Associated Grocery Manufacturers of America	1936
Gordon Wilson Lecturer and Medalist, American Clinical and Climatological Association	1939
Distinguished Service Award, American Medical Association	1945



# TREATMENT OF PERNICIOUS ANEMIA BY A SPECIAL DIET\*

By GEORGE R. MINOT, M.D., AND WILLIAM P. MURPHY, M.D.

THIS PAPER concerns the treatment in a series of forty-five cases of pernicious anemia in which the patients were given a special form of diet. While the problem of diet in the treatment of pernicious anemia is by no means new, in our opinion its possible importance has not heretofore been generally recognized. In 1863, seven years after the publication of Addison's second, but best known, description of the disease now called pernicious anemia, Habershon<sup>1</sup> wrote concerning this condition "Many patients at an early stage completely recover under the influence of bracing air and a nutrient and stimulating diet." Other early investigators of the disease, as Biermer<sup>2</sup> in 1872, and Pepper,<sup>3</sup> in 1875, appreciated the desirability of prescribing easily digested foods as a form of medication, but no greater emphasis was placed on the value of diet. Osler,<sup>4</sup> however, in 1885, mentioned that "cases [of pernicious anemia] appear to have got well with change of air and a better diet after resisting all ordinary means."

During the last half century, many clinicians, following the suggestions of the pioneer writers on the subject of pernicious anemia, have advised various kinds of diet as an aid to induce a remission of the disease. More often than not, the recommendations have been of a general sort as might be given for many persons with an impaired condition of the gastro-intestinal tract, which always is present in pernicious anemia. Thus, food for the pernicious anemia patient often has been selected because it appeared to be easily digested or because it seemed particularly nutritious and strength-giving. Rarely, diets have been chosen for some assumed direct effect on the blood.

The constant presence of achylia gastrica in pernicious anemia and the frequency of an abnormal bacterial activity within the intestines have been two main reasons for establishing certain forms of dietotherapy in the disease. On these accounts, Fenwick,<sup>5</sup> in 1880, and Naegeli,<sup>6</sup> among others, recommended diets relatively sparing in farinaceous foods and relatively rich in protein. For similar reasons, yet in contrast to the majority, Hunter,<sup>7</sup> in 1890, and others have advised quite the opposite type of diet. Grawitz<sup>8</sup> recommended a diet composed chiefly of fresh vegetables, followed by one with generous amounts of protein. The idea that forced feeding with any sort of food, but especially meats, is valuable to make weak and feeble individuals healthy and strong has caused the frequent use of this form of

From the Medical Clinic of the Peter Bent Brigham Hospital, and the Medical Service of the Collis P. Huntington Memorial Hospital of Harvard University.

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therapy in pernicious anemia, and Mosenthal<sup>9</sup> has shown that it can restore in these cases a positive nitrogen balance.

Meats and green vegetables, partly because of their iron content, have for a long time been thought to be useful to improve 'an anemic state of the blood.' Meat apparently has been chosen at times simply because it contained blood, which was supposed to be beneficial as food for persons who had an insufficient blood supply. The scientific foundations of the value of iron-containing foods to affect the blood-forming organs were laid by Menghini<sup>10</sup> in 1746, when he showed that iron could be increased in the blood by feeding such foods to animals. About 200 years later, Gibson and Howard<sup>11</sup> made important observations on the effect of a high iron content of the diet in anemia, and showed that in pernicious anemia it can have a most favorable influence on iron metabolism. They also showed that, in cases constantly losing nitrogen, a positive nitrogen balance could be obtained without forced feeding.

One thus finds that the diet usually advised for the pernicious anemia patient is one containing a relatively high nitrogen-content and often a relatively large number of calories. The recommendations of Smith<sup>12</sup> and of Barker and Sprunt<sup>13</sup> are of this sort and, like some others, the latter wisely recommend that the food be selected with a view to giving an ordinary, well balanced diet to replace a quantitatively deficient and qualitatively ill balanced one, on which these patients are apt to have placed themselves during their illness.

In spite of attention to diet for the anemic patient, the influence of food on blood formation and destruction has received comparatively little consideration, and special sorts of food, because of some particular effect, have seldom been chosen for patients with pernicious anemia.

Complete starvation in man is not considered to cause anemia, but may do so in animals. However, it is known that improper food can cause, and suitable food alleviate, anemia, for example, the 'iron starvation anemia' arising in infants who have partaken too long of only a milk diet, and who can be cured by food particularly containing complete proteins and iron. Incomplete diets, particularly those low in protein and relatively rich in concentrated carbohydrate food, can lead to anemia,<sup>14</sup> and even Shakespeare<sup>15</sup> recognized that improper food might impair the state of the blood. Likewise, patients with conditions due to, or associated with, vitamin deficiency experience anemia, and Jencks<sup>16</sup> has noticed that an abundance of vitamins favors blood regeneration. Certain foods, including liver, may benefit patients with sprue. This disease is considered by some partly dependent on a faulty diet, and resembles in numerous ways pernicious anemia, including the fact that the blood picture in the two diseases may be quite similar. Carnivorous animals and thin persons tend to have a greater percentage of hemoglobin in their blood than herbivorous animals and fat persons.<sup>17</sup> This further suggests, as do the observations of Morawitz and Kuhl<sup>18</sup> on man, the favorable role that animal protein food may play in blood formation, although dehydration may account for the differences observed.

Some of the earlier experimental work concerning the effect of food on blood

regeneration is reviewed by Pearce, Krumbhaar and Frazier<sup>10</sup> Adequate proteins as well as iron are necessary for the formation of hemoglobin Certain proteins will not suffice, such as gliadin<sup>20</sup> However, the amino-acid tryptophane may have a special ability to enhance blood formation<sup>21</sup> The most important recent work concerning the effect of food on blood regeneration has been done by Whipple and Robschert-Robbins and their associates<sup>22</sup> Their carefully controlled work on dogs has demonstrated clearly the value of certain foods, especially liver, on accelerating blood regeneration following acute hemorrhage and the value of iron added to the diet to decrease the anemia due to chronic blood loss

McCollum<sup>23</sup> has pointed out that liver and kidneys give an exceptionally high quality protein for a low protein intake and can enhance remarkably the growth of animals These foods are rich in nucleins, and Calkins, Bullock and Rohdenburg<sup>24</sup> have shown that the products of nuclein hydrolysis can stimulate growth Whipple<sup>25</sup> has suggested that in pernicious anemia there may be a scarcity of the material from which the stroma of the red blood cells are formed, or that a disease of the stroma-forming cells of the marrow exists Thus, theoretically perhaps liver and other foods rich in complete proteins may enhance the formation of red blood cells in this disease, especially by supplying material to build their stroma

Fresh red marrow was first used as a means of treatment for pernicious anemia by Fraser<sup>26</sup> in 1894 He reported beneficial results when a patient ate for some time about 100 Gm a day It was then and has since been given apparently on the supposition of some hormone effect Thus, numerous reports have appeared concerning the use of preparations of small amounts of concentrated bone marrow, but without definite evidence of advantage to the pernicious anemia patient Reports regarding the effect of eating generously of fresh marrow are few and brief, but suggest that it may be beneficial The nutritional composition of red bone marrow is similar to that of liver and kidneys If generous amounts of red marrow and liver can improve the state of the blood in pernicious anemia, may their influence not be due to the same, but unknown, cause?

Various investigators have commented on the blood-destroying properties of certain substances derived from fats, and the rôle they may play in pernicious anemia Stoeltzner<sup>27</sup> recently has reviewed the subject Also, lipoids have been shown by Baker and Carrel<sup>28</sup> to be a factor in serum that can inhibit growth Thus, founded on somewhat theoretical grounds, it seemed to us, as it did to Stoeltzner<sup>27</sup> and to Gibson and Howard,<sup>11</sup> that decreasing the amount of fat in the diet of the pernicious anemia patient might have a favorable effect on the state of his blood Excess of fat in a diet is considered by some to favor putrefaction within the intestine, a condition frequent in pernicious anemia Hence one might attribute any benefit derived from a low fat content of the diet to alterations in the bacterial flora rather than to some more direct effect on blood formation or destruction

A further hypothetic reason for decreasing the fat in the diet is that we have noted it is not uncommon for these patients to have consumed throughout life unusually large amounts of food rich in fats Patients with pernicious anemia also may give a history of partaking for years of some other type of one-sided diet It is common for them to do so after the definite onset of their illness, when it is not

unusual to find that they have a disgust for meat. Pernicious anemia is rare in certain parts of the world where diets are quite different (containing fewer dairy products, less free sugar and muscle meat) from those of the northern parts of Europe and America, in which areas the disease is relatively common. These different facts permit one to speculate on the possible partial rôle that some nutritional excess or deficiency may play in the etiology of the disease. Similar thoughts have occurred to others, including the idea that a vitamin deficiency might be a causative factor, as has been mentioned, for example, by Elders.<sup>29</sup>

Leafy vegetables and fruits usually are considered desirable for anemic patients, especially because of their iron content, and strawberries rich in iron appear beneficial for patients with sprue, a disease, as noted, resembling pernicious anemia. We prefer to add these foods to the pernicious anemia patient's diet not only because they are healthful ones for any person to eat, but also because, as Whipple and Robscheit-Robbins<sup>30</sup> have shown, certain ones have an especially favorable influence on hemoglobin production. It is quite probable, however, that their chief effect is not because of their iron content. It seems that such a factor as the character of the proteins or amino-acids in the diet is of much more importance than the iron content for pernicious anemia patients.

Numerous authorities hold the view that an intestinal bacterial toxemia plays an important etiologic rôle in this disease. One may choose to believe that any benefit these patients derive soon after beginning to take certain foods is to be attributed to changing rapidly the intestinal flora, thus decreasing a bacterial toxemia, rather than considering that the foods influence in some unknown, but more direct, manner the formation or destruction of red blood cells.

Gibson and Howard,<sup>11</sup> taking cognizance of Whipple and Robscheit-Robbins' work and the fact that certain lipid substances could enhance hemolysis, fed pernicious anemia patients a relatively low caloric diet (from 1,500 to 1,900) rich in iron [liver (daily), fruits, green vegetables, egg yolk] and low in fat and adequate in vitamins. A somewhat similar diet but containing a less amount of food rich in purines was recommended by Fenlon<sup>31</sup> in 1921. Gibson and Howard,<sup>11</sup> besides demonstrating the favorable influence of their diet on nitrogen and iron metabolism in pernicious anemia and some other anemias, suggested that it enhanced a remission in pernicious anemia and urged its use.

#### MATERIAL STUDIED AND OBSERVATIONS

Following the work of Whipple and Robscheit-Robbins, we made a few observations on patients concerning the influence of a diet containing an abundance of liver and muscle meat on blood regeneration. The effect appeared to be quite similar to that which they obtained in dogs. These observations, together with the information given above, led us to investigate the value of a diet with an abundance of food rich in complete proteins and iron—particularly liver—and relatively low in fat, as a means of treatment for pernicious anemia.

Observations set forth below have been made on forty-five patients with typical pernicious anemia first partaking of such a diet when in a relapse and continuing

it to date (except temporarily omitted by three), or from six weeks to two and a half years

The special diet<sup>32</sup> used was made as palatable as possible and for each day was practically as follows

- 1 From 120 to 240 Gm , and even sometimes more, of cooked calf s or beef liver. An equal quantity of lamb s kidneys was substituted occasionally
- 2 One hundred and twenty grams or more of beef or mutton muscle meat
- 3 Not less than 300 Gm of vegetables containing from 1 to 10 per cent of carbohydrate, especially lettuce and spinach
- 4 From 250 to 500 Gm of fruit, especially peaches, apricots, strawberries, pineapple, oranges and grapefruit
- 5 About 40 Gm of fat derived from butter and cream, allowed in order to make the food attractive. However, animal fats and oils were excluded so far as possible
- 6 If desired, an egg and 240 Gm of milk
- 7 In addition to the above mentioned foods, breads especially dry and crusty, potato, and cereals, in order to allow a total intake of between 2,000 and 3,000 calories composed usually of about 340 Gm of carbohydrate, 135 Gm of protein, and not more than 70 Gm of fat. Grossly sweet foods were not given, but sugar was allowed very sparingly

This diet is rich in iron and purine derivatives, containing about 0.03 Gm of the former and about 1 Gm of the latter

At the time the diet was advised for many of the patients, they were able to take only a small amount of food of any sort. Under these circumstances they were encouraged to take as much as possible of liver and fruits, and at least some vegetables, while other sorts of food were not forced. During the first week of the diet, the intake was often less than a thousand calories. After about this period of time, the patients usually felt distinctly better and their appetite began to improve. Then the food was increased gradually until the complete diet was taken. The patients as a rule did so within two weeks after the diet was begun. In fact, frequently they soon became "ravenously hungry" and often anxious to eat more than the customary allowance of liver and meat.

Twenty-four of the forty-five patients carried out the regimen by weighing portions of liver and meat and estimating the amounts of the rest of their food for at least three weeks, and often for the first six after commencing the diet. The other patients, like those after leaving the hospital, have taken their diet at home, following out written directions but not weighing any of their food. Our data strongly suggest that the patients who commenced treatment in the hospital and those few able to have a trained nurse at home have improved on the average rather faster and to an even better degree than the others. When the patients had remained much better for many weeks, their diet was sometimes modified particularly by decreasing the amount of liver and fruit.

The therapeutic regimen for these forty-five patients, besides the special diet, included rest, usually at first in bed for twenty-four hours a day. All but three also took each day about 15 cc of diluted hydrochloric acid (U. S. P.). These three, however, improved at least as much as the majority of the others. None of the patients received any especial treatment shortly before or after the diet was begun except as follows. A man, aged 69, with pronounced spinal cord lesions and ad-

vanced arteriosclerosis, was given five transfusions of blood within about six weeks while attempts were made to get him to eat. Now, three months later, he remains the least well of all forty-five, except for one woman who recently has omitted her diet. Blood was transfused to three others at about the time they first took the special diet. The red blood cell count of none was over 1,400,000 per cubic millimeter four days after transfusion.

The forty-five patients represent an essentially consecutive series seen in a relapse, and are all that have taken the special diet except one noted below. The series is not entirely consecutive, because during the time the forty-five cases were seen the following additional ones came under observation:

1. Four patients who had had their disease a long time were exceedingly sick, able to take little or no food, and died within a few weeks after they were seen. They ate no liver or kidneys.
2. Five patients consulting us but once and not taking the special diet. Letters indicate that three improved somewhat and two did not.
3. One patient that was in much better condition soon after taking the diet. This patient is not included in the series of forty-five because of several unusual complications.

Many of the forty-five patients had had definite symptoms due to pernicious anemia for more than two years, and two of them experienced such symptoms ten years before taking the special diet. A number of the cases were observed during a year or more before the diet was begun, others for several weeks, and some for only a few days. Many of the patients had remained in distinctly poor health and were unable to do their usual work for from a few months to more than a year before eating the food especially prescribed. During this time, many received various forms of therapy without distinct benefit, including transfusions of blood.

When the special diet was started, the forty-five patients that have continued to eat this kind of food fell naturally into the three following groups: (1) twelve in their first distinct relapse, (2) seventeen in their second relapse, (3) sixteen having had two or more relapses. It is thus evident that all sorts of variations of the disease occurred among the patients, and that the series was not composed chiefly of those in their first relapse, following which considerable "spontaneous" improvement is the rule.

The condition of all forty-five patients became much better rather rapidly soon after commencing the diet. All except one, who has recently omitted her diet, are now at the least in a very fair state of health, and if it were not for disorders in some due to spinal cord lesions, would have an appearance to a layman of being essentially well. However, there are only eleven patients who began the diet a year or more ago, two of whom have taken it for more than two years. Eighteen began taking the diet less than five months ago.

One of the earliest signs of improvement has been a change in the frequency of bowel movements believed to be due particularly to the diet and probably not to diluted hydrochloric acid. Within a few days, those who had had a tendency to diarrhea often began to have one formed stool a day, while, interestingly enough, those who had had normal movements or had been constipated frequently had for several days a few loose stools in each twenty-four hours. The latter patients, then, had a more natural regularity of their bowel movements and a more normal stool.

than they had had for some time before the diet was taken. The laxative effect of the diet has been observed also to occur in some normal persons.

Clinical improvement has been obvious usually within two weeks. This has been heralded in the peripheral blood before the end of the first week by the beginning of a most definite rise of the reticulocytes (young red blood corpuscles) of from about 10 per cent to usually about 80 and even to 155 per cent of all the red blood cells. This rise occurred in all fifteen patients that have had such counts made every day or so for from one to three weeks before and some weeks after beginning the diet. By the end of the second week, these cells usually had returned close to their normal percentage. Later, when the red blood cell counts were distinctly high, it

*Average Red Blood Corpuscle Count\**

Before diet started		After diet started					
		About 1 month		About 2 months		4 to 6 months	
		Number of cases	Average R B C counts	Number of cases†	Average R B C counts	Number of cases†	Average R B C counts
	millions		millions		millions		millions
19	0.90	19	3.28	15	4.08	12	4.50
15	1.60	15	3.25	13	4.09	10	4.54
11	2.30	11	3.83	9	4.41	5	4.47
45	1.47	45	3.40	37	4.16	27	4.50

\* The figures represent the count per cubic millimeter before and after starting special diet in three groups of cases of pernicious anemia (1) with less than 1.2 million, (2) having from 1.2 to 2 million, and (3) having from 2 to 2.75 million before diet was begun. Also, averages for all forty-five cases are shown.

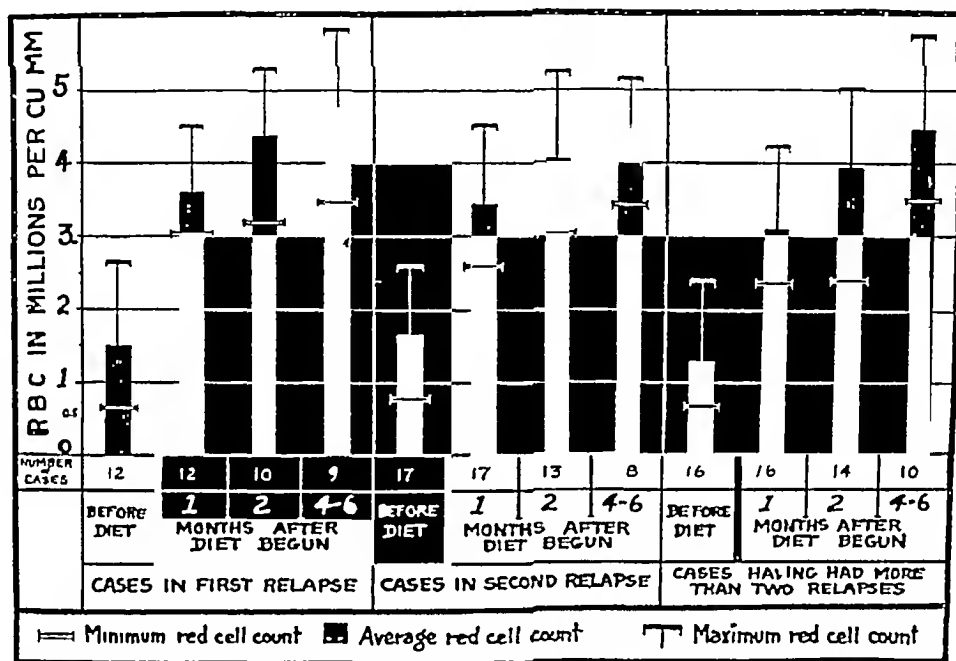
† The differences in the number of cases after about one month is because some have not taken the diet for as long as two, and others as long as four months.

was frequent to find, as we have noted formerly, an abnormally small number of reticulocytes. Before they began to increase, the icterus index of the blood serum in these fifteen patients started to fall, and soon the yellow tint of the patient's skin disappeared. This index reached normal in from two to four weeks, and often has fallen to below normal even when the red blood cell count had increased to only 2,500,000 per cubic millimeter.<sup>33</sup>

The accompanying chart and table give in a synopsis manner the trends of the state of the blood in the forty-five patients, taking into consideration, on the one hand, the character of the case, and, on the other, the level of the red blood cells when the diet was begun. The data are given for all forty-five patients before and about one month (from four to six weeks) after the diet was started. Although all the patients have been observed repeatedly, data can be given for only thirty-seven at the end of about two months (from eight to eleven weeks) of treatment, and for twenty-seven between four and six months after treatment began, because eight have taken the diet for less than two months, and eighteen for less than four months. As a measure of the patient's condition, we have chosen to give in the table and chart the red blood cell count rather than the hemoglobin percentage, partly because the latter in pernicious anemia may be at about the same high level

(80 per cent) with red blood cell counts of from 2.5 to 4 million per cubic millimeter. It is recognized that figures for both may vary considerably within a few hours. The figure used in synthesizing the data often represents in each instance an average of several counts made within a few days of each other.

Inspection of the chart and table shows the rapidity with which the red blood corpuscles increased, the high level they attained at the end of about one month, two months and from four to six months after the diet was begun, and the rather slight differences that occurred in the bloods in the cases falling into the three groups based on the number of relapses that had occurred. The percentage increase of cells (and the same is true of the hemoglobin) at the end of a month was usually very



Red blood cell counts in forty-five cases of pernicious anemia before and after beginning special diet. Cases grouped according to the number of relapses the patients had had. One and two months after diet began indicates an approximate amount of time and for any given case is not less and often somewhat more than four or eight weeks. The differences in the total number of cases after about one month are caused by the fact that some patients have had the diet for less than two, and others for less than four, months.

much greater in patients starting the diet when their red blood cell count was less than 1,200,000 per cubic millimeter than in those in whom it was distinctly higher. This occurs in other pernicious anemia patients rapidly restoring their blood. The blood of patients with rather high counts of their red blood corpuscles and prominent signs of injury to the spinal cord responded more slowly perhaps and less well than others, and, as is to be expected, no striking change occurred in very marked symptoms or signs due to spinal cord degeneration.

In pernicious anemia, remissions after two relapses are frequently less marked than previous ones, so that the red blood cell count is apt to be lower in a third or subsequent relapse than in a former one. In spite of the excellent remissions our patients had soon after beginning the diet, the data in the chart show what might



be expected, namely, that not only did the third group of patients (those having had more than two relapses) have on the average a slightly lower red blood cell count before the diet was started, but also that afterward their counts were apt to increase more slowly and not become quite so high as in the other two groups. Only four of the patients had red blood cell counts as low as between 3 and 2.5 million per cubic millimeter after taking the diet for about a month. The cases of three belong to this third group. Even so, two had 4,000,000 or more red blood cells per cubic millimeter at the end of four months. The other, the patient transfused several times, has now, after three months of dieting, only 2,600,000 per cubic millimeter. However, his hemoglobin has risen from 25 to 70 per cent. The fourth case with a red blood cell count of less than 3,000,000 per cubic millimeter at the end of a month belongs to the second group, and now, two and a half months after the diet was started, shows a red blood cell count of 3,300,000 per cubic millimeter.

The data from which the table and chart were prepared have been analyzed in various ways, and the following statements indicate in a different manner than they do what satisfactory improvement was shown in the patients' blood. Seventy-six per cent of all the patients had 2,000,000 or less red blood corpuscles per cubic millimeter, with their hemoglobin usually 55 per cent or less before beginning the diet. In contrast to this, approximately a month (from four to six weeks) later, 91 per cent had over 3,000,000 and 42 per cent over 3,500,000 red blood cells per cubic millimeter, with corresponding rises in the hemoglobin percentage. After taking the diet for about two months (from eight to eleven weeks), 89 per cent (of the 37 that had taken the diet this length of time) had 3,500,000 or more red blood corpuscles per cubic millimeter, while 73 per cent had 4,000,000 or more. All had a hemoglobin of approximately 80 per cent or over. None of the patients studied after they had eaten the food selected for them for between four and six months had less than 3,500,000 red blood cells per cubic millimeter, 81 per cent had 4,000,000 or more, and the counts of 30 per cent were over 5,000,000 per cubic millimeter. The hemoglobin was 80 per cent or above in all, often 90 per cent, and in several cases reached more than 100 per cent. However, it is to be noted that none of these cases observed between four and six months after the diet was started had appeared as advanced as several of those in patients that improved the least, but which have not yet had the diet for four months. The observations on the eighteen patients who have been on the diet for more than six months show that their count may fluctuate, though it has remained above 3,200,000 per cubic millimeter, and usually has been found over 4,000,000, with the hemoglobin remaining 80 per cent or more. There are three exceptions to this statement, for three patients had a relapse about eight weeks after changing their diet. One did so a year and another seven months after the special diet was begun. Both had for two or three weeks a count slightly below 3,000,000 per cubic millimeter. Their red blood cells and hemoglobin then very rapidly increased under rest and on eating an increased amount of liver and fruit. The third patient's red cell count was 4,200,000 per cubic millimeter a month before she changed her diet. She has just resumed the special diet, and her red cell count is 1,900,000 per cubic millimeter and hemoglobin 50 per cent.

## COMMENT

Cases of pernicious anemia undergoing distinct remissions often show rapid and striking improvement, such as occurred in almost all our patients. A considerable number of them have made such remarks as, 'I feel better than for several years,' 'better than for two years,' and 'stronger than after the two times my blood went low before.' Such statements, to be sure, are made by pernicious anemia patients having remissions that have not taken this diet, and there is no case in this series of forty-five that cannot be paralleled by a similar one having a so-called spontaneous remission. However, the records of eleven cases show that the red blood cell count in the remission following the 'liver diet' has remained distinctly higher, not only than in a former remission, but also in three cases higher, for at least two months, than in their three previous remissions. It is, thus, again pointed out here that it is rather unusual to find the red blood corpuscle count in a late remission distinctly above the level obtained in several earlier ones. A few of the patients observed for many months before they took the special diet ate, by our advice, relatively small amounts of liver two or three times a week, together with other food of the sort contained in the special diet. Under such a regimen a moderate degree of improvement occurred in some, to be followed later by a relapse of their case. It was rather striking that when the same patients were placed on a diet rich in liver they improved markedly. This suggests, as do similar observations we made some time ago in other cases, that if liver and food like it play a role in improving the blood of pernicious anemia, it is desirable for the patients to take such food daily and in large amounts.

The spontaneous remissions of pernicious anemia and the bizarre course it often runs make it notoriously difficult to determine accurately the effect of any procedure on the disease. All sorts of therapeutic procedures have been advised, many because a few cases improved promptly after their trial. Waves of enthusiasm for certain methods have vanished soon when it was shown that the earlier reports of benefit could be attributed readily to the natural course of the disease. There is, however, no doubt, as shown by some of the early and more recent investigators, that a well balanced, nutritious diet sometimes aids to enhance a remission. The patient may be helped by numerous other forms of treatment, such as those to change the intestinal flora, the injection of protein substances, the taking of arsenic, the transfusion of blood, and splenectomy.

At least one remission, as has been noted by Cabot,<sup>34</sup> takes place at some time, but at no regular time, in about 80 per cent of pernicious anemia cases. Precise data are sparse concerning the frequency, degree and rate of remissions in similar groups of cases treated in different ways. Splenectomy has caused quick and marked improvement in 64 per cent of the patients undergoing this operation, while about 15 per cent more have shown some benefit from the procedure.<sup>35</sup> However, the remissions that followed have been of no longer duration than those heretofore reported as of a spontaneous nature. Excluding desperately ill patients, Minot and Lee<sup>36</sup> noted in 1917 that about 35 per cent of forty patients treated in no especial manner had a moderate or better remission soon after they were seen. Following the transfusion

of blood into forty-six similar patients, about 50 per cent continued to have definitely improved health for at least many weeks than for some time before the procedure. Not more than 20 per cent of the ninety-six patients of these two groups soon had rapid and marked increase in their red blood cells. An analysis of fifty other cases observed between 1916 and 1923 in sequence, except for several in a terminal condition, indicates that 45 per cent developed a definite remission soon after we saw them. These patients were treated in various ways by numerous physicians, but did not eat large amounts of liver or similar food. The remissions were seldom of marked degree, with the red blood corpuscles reaching 4,000,000 or more per cubic millimeter.

No entirely satisfactory data have been found concerning the frequency of remissions following the use of a nutritious high caloric diet and such a regimen as that prescribed by Barker and Sprunt. We have treated in this manner twenty-five partially selected cases, from which it appears that distinct remissions may follow such therapy in about 65 per cent of the instances. Even so, apparently the red blood cell counts of patients on such a diet and who were improved distinctly at the end of one or two months averaged less than for all forty-five who have eaten generously of liver for the same amount of time.

The evidence at hand suggests that the dietetic treatment of pernicious anemia is of considerable importance. It has been possible to demonstrate in forty-five cases seen essentially in sequence that following a diet rich in liver and low in fat a distinct remission of the anemia occurred rather promptly. The promptness and rapidity with which the red blood corpuscles and hemoglobin increased, coincident with at least rather marked subjective improvement in the sense of well being and clinical appearance of all the patients and the strikingly better health of many, is at least unusual in pernicious anemia. It is also not customary for the red blood cell counts during remissions of pernicious anemia to be so frequently of the height that occurred in these patients. We are inclined to believe that something contained in the foods rich in complete proteins is particularly responsible for the improvement in the state of the blood. The low fat content of the diet is assumed to have a less important effect than the character and amount of protein, although probably excess of nitrogen per se is unimportant. If liver and similar food is of value, every means must be taken, including the skill of the nurse and cook, to get patients to eat daily as much as possible, preferably 200 Gm. or more. Failure could be attributed to taking too little of such food.

There are no data to indicate whether the remissions in these forty-five cases will last longer than those of others.

It is possible that this series of cases eventually may be proved to be unusual in that there happened to be treated a group that would have taken a turn for the better under other circumstances. Also, time may show that the special diet used, or liver and similar food, is no more advantageous in the treatment of pernicious anemia than any ordinary nutritious diet. Let this be as it may, at the present time it seems to us, as it has to Gibson and Howard, that it is wise to urge pernicious anemia patients to take a diet of the sort described.

## SUMMARY

The dietetic treatment of pernicious anemia is of more importance than hitherto generally recognized

Forty-five patients with pernicious anemia observed essentially in sequence are continuing to take a special diet that they have now been living on for from about six weeks to two years but which was temporarily omitted by three. This diet is composed especially of foods rich in complete proteins and iron—particularly liver—and containing an abundance of fruits and fresh vegetables and relatively low in fat

Following the diet, all the patients showed a prompt, rapid and distinct remission of their anemia, coincident with at least rather marked symptomatic improvement, except for pronounced disorders due to spinal cord degeneration. Improvement was often striking, so that where the red blood cell count averaged for all before starting the diet 1,470,000 per cubic millimeter, one month afterward it averaged 3,400,000, and for the twenty-seven cases observed from four to six months after the diet was begun, the average count was 4,500,000 per cubic millimeter

Patients having had two or more relapses showed on the average slightly lower red blood corpuscle counts about one and two months after commencing the diet than did those who had started it in their first or second relapse

Change in the frequency of bowel movements, temporary increase of reticulocytes in the peripheral blood, and decrease of the icterus index of the blood serum were among the earliest signs that heralded the patient's better health

All the patients have remained to date in a good state of health except three, who discontinued the diet, two rapidly improved on resuming it and the other has just commenced it again. As the diet was advised for most of the patients less than eight months ago, enough time has not yet elapsed to determine whether or not the remissions will last any longer than in other cases

## SUBSEQUENT OBSERVATIONS

Since the data presented in this paper were compiled, the following additional information has been obtained. The eight patients who had taken the diet for only about one month had red blood cell counts at the end of about two months of between 3,500,000 and 6,000,000, with an average of 4,400,000 per cubic millimeter. One of these had but 2,500,000 at the end of one month and now at the end of three and a half months has 4,500,000 per cubic millimeter

The ten patients recorded as having taken the diet for only about two months showed in four to six months after starting it as follows. Seven had an average red blood cell count of 5,100,000 per cubic millimeter. One who had had about 5,000,000 had but 3,500,000 per cubic millimeter. Another who had 2,500,000 per cubic millimeter at the end of the second month had the same number two months later, although symptomatically he seemed better. The tenth patient could not obtain the proper diet between the second and fourth month, and had at the latter time 3,000,000 per cubic millimeter

The patients who had the diet from four to six months, or longer, when the data were compiled, continued in the next two and a half months to have on the average as satisfactory counts, except as noted below. The majority of these have shown higher counts than formerly. Two of the cases have had at three different times red blood cell counts of 6,000,000 or more per cubic millimeter. Two patients who have had the diet for more than six months have recently eaten very little liver, and their counts have fallen in two months from about 4,000,000 to about 3,000,000 per cubic millimeter. The red blood corpuscles of the patient referred to on page 16 as in a relapse increased 3,000,000 per cubic millimeter during the first eight weeks after the diet was resumed.

Information at hand suggests that some cases in which transfusion is done many times before the diet is started may respond but little to it.

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## PERNICIOUS ANEMIA FROM ADDISON TO FOLIC ACID\*

By RUSSELL L. HADEN, M D

A CONSTANTLY fatal disease unexplained at autopsy is always intriguing. The mysterious nature of pernicious anemia thus interested Thomas Addison<sup>1</sup> when he described the first group of patients in 1849. He said this is "a remarkable form of anemia which has not attracted the attention it really deserves." The anemia was profound and of unknown origin. The patient became progressively weaker with little wasting and finally died without response to any treatment. A postmortem examination did not aid in explaining the problem. No real progress was made in solving the puzzle until the discovery of the beneficial effect of liver feeding in 1926 by Minot and Murphy<sup>2</sup> completely altered the outlook of the patient. Further research is slowly unraveling the mystery. Clinicians still think, however, of pernicious anemia as a "remarkable form of anemia."

It is my purpose to discuss historical highlights of this interesting disease from the time of Addison to the discovery of folic acid, and to emphasize some important clinical aspects.

Idiopathic pernicious anemia is a disease of nutrition characterized by macrocytic anemia, histamine-refractory achlorhydria, combined sclerosis of the spinal cord, and a specific response to liver and liver substitutes. The anemia alone may be completely relieved by a single chemical compound, pteroylglutamic acid (folic acid). The clinical picture is variable, the anemia may be minimal, only about three-fourths of the patients have signs of a cord lesion initially, a loss of vibratory sense is usually the earliest and often the only evidence of neurologic involvement, achlorhydria is a constant finding.

It is a disease of older people. In 427 patients studied at the Cleveland Clinic only 5 were less than 30 years of age. In a total number of 579 I have seen, the anemia began in only 1 individual less than 20 years of age. Fifty-two per cent of the patients were between 40 and 60. A very large proportion were over 60 when the diagnosis was made.

Numerous clinicians, beginning with Combe in 1822,<sup>3</sup> reported fatal unexplained cases of anemia which we now recognize as pernicious anemia. Thomas Addison, however, first in 1849 and again in 1855<sup>4</sup> described it as a clinical entity.

Why was the disease so-called? Addison in his original description speaks of it as "a remarkable form of anemia." Its approach is first indicated by a certain amount of languor and restlessness to which presently succeed a manifest paleness of the countenance. The symptoms go on increasing. The patient experiences a distressing and increasing sense of helplessness and faintness. He dies either from sheer exhaustion or death is preceded by signs of passive effusion or cerebral oppression." All patients in this group were not suffering from true pernicious anemia since 2 recovered and in 3, disease of the adrenal was found at autopsy. Addison said in 1855 that he was trying to throw additional light on this

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\* Peter T. Bohan lecture given at the University of Kansas Medical School, March, 1947

condition when he discovered the disease of the adrenal glands known as Addison's disease. He again emphasized that there was no "discoverable cause whatever."

Addison recognized the anemia only by the pallor of the skin and the thinness of the blood. In 1849, no blood cell counts or hemoglobin estimations had been done. Vierordt<sup>5</sup> did the first red cell count in 1851, Funke<sup>6</sup> discovered hemoglobin the same year, and Welcher<sup>7</sup> published the first extensive clinical article reporting blood counts and hemoglobin estimations in numerous diseases in 1854. Thus, accurate measurements of the blood came after Addison's original communication.

Addison's observations made little impression even in England, and little more was heard of this "remarkable anemia" until it was reported independently by Biermer<sup>8</sup> in Switzerland in 1871. At a meeting of the Medical Society in Zurich, November 6, 1871, Biermer under the title of Progressive Pernicious Anemia described 15 cases of severe anemia. He used the name only in a symptomatic sense, grouping together anemias of widely different etiology. He had previously<sup>9</sup> mentioned similar cases in which he emphasized fatty degeneration of the heart and vessels. Biermer stressed the role of pregnancy. So some cases he described were evidently what we know now as the anemia of pregnancy. His group has been described as a provisional shelter for a multitude of cases.<sup>10</sup> He did not think of pernicious anemia as a single disease. He again emphasized the finding at autopsy of fatty degeneration of the heart muscle and small vessels.

Biermer's report was published in 1872 in the proceedings of the Medical Society in Zurich. For some unknown reason it quickly excited the interest of clinicians everywhere. Articles on progressive pernicious anemia began to appear rapidly. In England, Addison's original description was not recalled until stimulated by Biermer's work. In 1875, William Pepper<sup>11</sup> in Philadelphia wrote an extensive article of 26 pages on the disease in the *American Journal of Medical Sciences*. Pepper says, "My present purpose is to offer a contribution to this important study by calling attention to a peculiar form of anemia of obscure and fatal character which has recently been redescribed (i.e. by Biermer) as though it were a new affection under the name of Progressive Pernicious Anemia." He then emphasizes that Addison had previously described the disease as "idiopathic anemia." Pepper's main contribution is his discovery of the extreme hyperplasia of the marrow. He considered pernicious anemia as a primary disease of the bone marrow.

Many papers on the subject were published between 1875 and 1878. In 1878, Eichhorst's extensive monograph<sup>12</sup> of 375 pages entitled "Progressive Pernicious Anemia" appeared. All cases previously reported were reviewed. Eichhorst mentioned Addison's work but gave him little credit. "Addison," he said, "considered the anemia due to fatty degeneration of the internal organs while we now know that the anemia is primary and the fatty degeneration is secondary." Eichhorst described as pernicious anemia cases of anemia which we now exclude. The term designated only a group of fatal anemias and included such conditions as true aplastic anemia, leukemia, and other bone marrow diseases as well as severe anemias due to infection and toxemia. Eichhorst did not have our present concept of pernicious anemia as a single specific entity. The name he used, however, has persisted. Interest stimulated by these early papers has never abated.



Pernicious anemia is defined as a macrocytic anemia—the red cells are characteristically large. Eichhorst mentions macrocytosis but reports no measurements or even counts in his own cases. He does say the number of red cells was about one-tenth or one-twenty-fifth of normal. The first blood count in a patient with pernicious anemia seems to have been done by Sørensen<sup>13</sup> in 1874 when he counted the blood with Malassez's apparatus and found only 470,000 red cells. Sørensen also emphasized the large size of the cells. The diameter of red cells had been measured from the time of Leeuwenhoek.<sup>14</sup> A monograph on the dimensions of red blood corpuscles by Manassein<sup>15</sup> had appeared in 1872. Eichhorst concluded that the diameter of the cells is almost always increased.<sup>16</sup> Laache in his book on the anemias<sup>17</sup> published in 1883 has a long discussion of pernicious anemia and emphasizes the large size of the red cells and the increased color index. The decrease in number of red cells, the increase in size, and the increase in hemoglobin content were thus established very early as characteristic findings.

Earlier workers used the red cell diameter as a measure of size. With the development of the hematocrit the cell volume was found increased also and a more sensitive indicator of macrocytosis. Capps,<sup>18</sup> in his work on volume index, found this always increased in pernicious anemia. In our series of 579 patients all showed a macrocytosis if untreated except in the rare instance with a coincident iron deficiency. Other clinical conditions will also produce a macrocytosis but seldom so marked as in a pernicious anemia. Examples are liver disease, intestinal obstruction and nutritional deficiency such as sprue. Ehrlich considered the presence of megaloblasts in the peripheral blood as diagnostic of pernicious anemia. He insisted that these were pathologic nucleated red cells and not simply very young cells. Although Ehrlich and others believed that the diagnosis of pernicious anemia should not be made without the finding of megaloblasts in the blood, this view is no longer held. A diagnosis should never be made of untreated idiopathic pernicious anemia in the absence of a macrocytosis of the red cells.

The presence of an achlorhydria refractory to histamine stimulation is an essential finding. All clinicians now accept the fact that idiopathic pernicious anemia should never be diagnosed if free hydrochloric acid be present on gastric analysis.\* The achlorhydria has been a most important factor in the final solution of the origin of the disease. Addison and Biermer knew nothing about achlorhydria.

How did it become recognized that this was a necessary part of the symptom complex? Test meals were not done until relatively late in clinical medicine. Cahn and von Mering<sup>19</sup> first studied the acid in healthy and diseased stomachs in 1886. During the next ten years many articles on the subject appeared in England, on the continent, and in this country. It was soon noted by numerous investigators that when no free hydrochloric acid was found the patients were frequently anemic, and that the anemia belonged in the group already designated as pernicious. As late as 1900, however, Faber and Bloch<sup>20</sup> could collect only 33 cases of pernicious anemia on whom a test meal had been done. Martius and von Lubarsch,<sup>21</sup> in the first monograph on achylia gastrica in 1897, reported both pernicious anemia and

\* Wilkinson and Israëls, Waldenström and others report that achlorhydria occurs in approximately 1 case of 100 *Eds*.

secondary anemia associated with achlorhydria. Achlorhydria was not then considered as a necessary part of the clinical picture. The first large group of patients with pernicious anemia on whom test meals had been done were reported by Levine and Ladd<sup>22</sup> in 1921. In 107 patients only 3 were found to have free acid. In 2 of these 3 patients the diagnosis was questioned. In the light of present day knowledge all would be questioned. One, for instance, had had several operations and a persistent diarrhea following an intestinal resection. This patient probably had a symptomless obstruction of the small bowel with a macrocytic anemia. Recently Goldhamer,<sup>23</sup> in a report on the gastric acidity during remission in pernicious anemia, mentions 1000 patients at the Simpson Memorial Institute as having had a test meal without finding free hydrochloric acid in a single one. In our series of 579 patients a test meal was done in 546. Free acid was found but once. This patient had a typical clinical and blood picture of pernicious anemia with subacute combined sclerosis. A technical error was not excluded. The test meal was not repeated because the patient died soon after the original examination. No special studies were done to exclude other causes for a macrocytic anemia. Recently we have studied 2 patients with a macrocytic anemia and a normal gastric analysis. Both were found to have a benign chronic intestinal obstruction. These 2 patients also had signs of a subacute combined sclerosis.

A possible relation of the stomach to pernicious anemia through impaired nutrition was recognized long before test meals were done. Immerman<sup>10</sup> in 1877 described pernicious anemia as a disease of nutrition due to faulty absorption of food. Austin Flint<sup>24</sup> in 1860 said, "Nor is it difficult to see how fatal anemia must follow an amount of degenerative disease reducing the amount of gastric juice so that the assimilation of food is rendered wholly inadequate to the wants of the body." The English physician, Samuel Fenwick, especially emphasized this point of view. His book, "Atrophy of the Stomach,"<sup>25</sup> was published in 1880. Here he recognized severe anemia as occurring with atrophy of the stomach. In Chapter 3 on "The Relation of Gastric Atrophy to Other Forms of Idiopathic Anemia" he remarked that the cases of atrophy of the stomach with anemia reported by him were identical with those described by Addison as idiopathic or pernicious anemia. He quoted Addison's description to emphasize the similarity. Fenwick thought, however, that the anemia was produced by interference with nutrition. He pointed out that the digestive powers of the stomach were so impaired that the usual postmortem digestion solution of the gastric mucosa did not even take place unless acid were added, and the gastric contents would not digest egg albumin. These observations of Fenwick are most important in the light of present knowledge of the relation of the stomach to pernicious anemia. This atrophic condition of the gastric mucosa in pernicious anemia can now be verified in life by gastroscopy.

William Hunter<sup>26</sup> long emphasized the relation of the digestive tract to pernicious anemia. He considered the gastric atrophy as resulting from a gastritis due to swallowing bacteria, and the characteristic glossitis to be produced by a specific micro-organism. He believed that a toxin of bacterial origin in the intestinal tract was absorbed into the portal blood and destroyed red cells.

The earlier students of pernicious anemia did not recognize central nervous

system involvement In 1887, Lichtheim<sup>27</sup> described 3 patients with severe anemia and involvement of the central nervous system Lichtenstein<sup>28</sup> in 1884 had previously described cases of pernicious anemia with findings suggesting tabes dorsalis We now think these patients had pernicious anemia with subacute combined sclerosis In 1892, Minnich<sup>29</sup> described 2 patients with pernicious anemia who had serious cord involvement and studied the cord at autopsy He found changes especially in the posterior columns of the spinal cord In this country Dana<sup>30</sup> in 1891, in a discussion of degenerative diseases of the spinal cord, described a case with extreme anemia and diarrhea which was evidently pernicious anemia with cord involvement In the same year Putnam<sup>31</sup> described 8 patients with combined sclerosis which we recognize as having pernicious anemia from the characteristic anemia and other symptoms It is interesting that few of the early observers did blood counts on their patients, so the anemia was evidently quite extreme to be recognized only by pallor or weakness These observers continually emphasized that the nerve involvement is due to poor nutrition resulting from the anemia After 1890, following such early reports numerous articles appeared describing cord lesions In 1902, McCrae<sup>32</sup> reported 50 patients with pernicious anemia from the Johns Hopkins Hospital and found neurologic manifestations in 20 of these In 1900, Frank Billings<sup>33</sup> took as his subject for the Shattuck Lecture in Boston, 'The Changes in the Spinal Cord and Medulla in Pernicious Anemia' He emphasized the now well established relation of diffuse cord degeneration and pernicious anemia He thought the anemia and cord changes resulted from a simple toxin which was probably of intestinal origin His article is illustrated with many sections of spinal cord obtained at autopsy

Russell, Batten, and Collier<sup>34</sup> in 1900 in discussing subacute combined degeneration of spinal cord described this condition as occurring in patients with severe anemia which was evidently pernicious anemia They thought there was no etiologic relation of the anemia to cord changes In the earlier articles there is necessarily much confusion since the criteria for the diagnosis were not clear Many diagnoses were missed and often cases of severe anemia due to other causes were called pernicious anemia

The central nervous system is affected in 80-85 per cent of cases of true pernicious anemia The most common evidence of cord involvement is a diminution of vibratory sense The cord lesion may be the only significant manifestation of the disease, it may be more serious than the anemia There is no parallel between the degree of anemia and involvement of central nervous system Subacute combined sclerosis may arise from other causes The cord lesions usually respond, at least partially, to liver therapy Sometimes the damage to the central nervous system is beyond repair, so neurologic symptoms and signs may persist when the anemia is entirely relieved

The proof of the relation of the stomach to the origin of pernicious anemia is a most important discovery It is easy to see how the stomach was early incriminated since the anemia had been conceived of as a wasting disease due to impaired nutrition This was well expressed by Austin Flint<sup>24</sup> in 1860, as already quoted Immerman's classification of pernicious anemia as a disease of nutrition, and Fenwick's

work, begun in 1871, on gastric atrophy as a cause of anemia, have already been mentioned. Henry and Osler<sup>35</sup> in 1886 described a case of pernicious anemia as due to gastric atrophy. Numerous other clinicians made similar reports. Pepper,<sup>11</sup> in his very complete article, however, lays no emphasis on changes in the stomach. Then, as gastric analyses were more widely employed, came the discovery that patients with pernicious anemia had an achlorhydria, and finally the conclusion of all clinicians that achlorhydria is invariable in the idiopathic form of the disease. Achlorhydria usually, if not always, precedes the development of the anemia by many years, and persists even in complete remissions. Free hydrochloric acid is not only absent in idiopathic pernicious anemia but the amount of gastric secretion is greatly decreased. Askey<sup>36</sup> has recently reviewed 47 cases of pernicious anemia reported as showing free hydrochloric acid on gastric analysis. He emphasized that none can be considered true Addisonian pernicious anemia by present day criteria.

What is the relation of achlorhydria to the causation of pernicious anemia? We are indebted to Castle<sup>37, 38</sup> for the proof that achylia gastrica is a necessary link in the development of the nutritional deficiency producing the disease. He showed that a patient fails to secrete in the stomach some unknown substance, probably a ferment, which acts on the food to produce a substance or substances necessary for the maturation of the red cells in the bone marrow and for the normal metabolism of nervous tissue. The proof is simple. Ground beef partially digested in the stomach of a normal man with normal gastric secretion when fed to a patient with active pernicious anemia initiates a remission, and causes active blood formation as shown by a rise in reticulocytes and increase in red cells and hemoglobin. Similar preparations exposed to digestion in the stomach of a person with pernicious anemia cause no reticulocytosis or erythrocytosis in other patients suffering from pernicious anemia to whom the material is fed. Such observations proved that a substance supplied by gastric mucosa is a necessary link in the protection against pernicious anemia. It was also shown that the achlorhydria by itself is not a factor but the ferment is never absent if free hydrochloric acid is present. On the other hand the specific ferment may be present if free hydrochloric acid be absent. Castle's work furnished the final proof that pernicious anemia is a deficiency dependent primarily on a gastric defect. Many workers, such as Austin Flint, were right in considering the absence of normal gastric digestion as a cause of anemia though they never thought of such specific action as that demonstrated by Castle. Pernicious anemia may follow total gastrectomy. Meulengracht<sup>39</sup> thinks Brunner's glands in the duodenum supply the specific ferment also. If true, this explains normal blood formation after some cases of gastrectomy.

Castle's work disproved other theories of pernicious anemia. Gastrointestinal toxemia, infection, and other possible causes are no longer mentioned. The disease becomes a negative one due to the lack of something, and not a hemolytic one, due to the action of some positive toxic agent.\*

The discovery of a specific treatment for pernicious anemia is the most dramatic episode in the long history of this serious disease. Many different methods of

\* However, a hemolytic component, causation obscure, is usually present. *Eds*

treatment had been used prior to 1926—iron, hydrochloric acid, arsenic, transfusion, splenectomy, removal of infection, special diets, and drainage of the intestinal tract. At times any method of treatment seemed to produce a remission. Sometimes the effect of transfusion was lifesaving by initiating a remission. No treatment, however, could be depended on to stay permanently the course of the anemia. It was almost always progressive, and usually ended in death from anemia unless some intercurrent fatal disease developed.

In 1920, Whipple<sup>40</sup> and his associates had begun a systematic study of the effect of different methods of treatment, especially food and drugs, on experimental hemorrhagic anemia in the dog. They found that the most valuable agent in ameliorating the anemia was whole liver. Other foods, such as red meat, had a similar effect to a varying degree but the effect was not so striking as with liver. While Whipple was working with hemorrhagic anemia he emphasized in 1925<sup>41</sup> that "even in complex anemia such as pernicious anemia, anemia with nephritis, and cancer cachexia food factors deserve serious consideration in the clinical management of the blood conditions." Whipple did not apply his discoveries to clinical medicine, however. It remained for Minot and Murphy<sup>2</sup> to find in a routine trial of liver in various types of anemias, that the response in pernicious anemia was strikingly different from that in other types of anemia. They were helped by the knowledge that the level of reticulocytes is an ideal method of gauging response to treatment. Minot and Murphy's discovery was first announced in 1926 and was rapidly verified by clinicians everywhere.

Liver and liver extracts affect the stroma of erythrocytes only. This verified Whipple's idea expressed in 1922<sup>42</sup> that there is a scarcity of stroma-building material in pernicious anemia. With adequate liver therapy the blood of a patient about to die rapidly responds and returns completely to normal. The glossitis and other gastrointestinal symptoms disappear entirely. The neurologic symptoms become improved or do not progress, at times they disappear entirely. There is nothing more dramatic in medicine than the effect of liver therapy on a patient with pernicious anemia. Only the use of sulfa drugs and other antibiotics such as penicillin afford such brilliant results.

It was soon found that a liver extract acted just as well as whole liver. Extracts have been improved until now these are almost perfect in their action. A monthly injection of a potent extract will keep the blood normal and prevent the development of a neurologic lesion. An extract of normal gastric mucosa has a similar action as one would expect from Castle's discovery.

Many attempts have been made to isolate from liver and liver extract a single specific substance responsible for the beneficial effect. While highly concentrated preparations have been made no single substance has been isolated. In the meantime, a single chemical substance has been found which gives a specific blood response in pernicious anemia and related macrocytic anemias. Folic acid, a substance found in liver, yeast, spinach, and grasses, has proved to be necessary for the growth of certain bacteria and to relieve the anemia developing in certain vitamin deficient diets. This substance was found to be effective in macrocytic anemias due to a deficiency such as sprue, and other related conditions.

Folic acid is a single chemical compound (pteroylglutamic acid) which causes a specific response also in pernicious anemia. It matures the megaloblasts of the bone marrow so that the blood returns to normal. It probably has little effect on the cord lesion. The latest reports indicate a cord lesion may even develop while the anemia is disappearing and the blood count is normal.

With folic acid the reticulocyte response is not so pronounced as with a potent liver extract but the full effect is excellent and the blood will return to normal. No secondary reticulocytosis occurs in a patient treated with folic acid when liver extract is added. The question is still unsettled whether liver extract and folic acid give a better result than liver extract alone. Folic acid alone should never be used in the treatment of pernicious anemia since it is not the antineuritic factor. It fails to prevent the development or progression of neurologic symptoms indicative of subacute combined sclerosis.<sup>43</sup>

The anemia of pernicious anemia is due to the lack of a specific red cell maturing factor necessary for the normal development of the erythrocyte. This may well be folic acid since the response of the anemia with adequate amounts of folic acid is complete. The relation of liver extract to folic acid is now being investigated. Liver extract contains small amounts of folic acid but not enough to explain its anti-anemic action. Liver extract has a widespread effect on the individual needing it, possibly through its action on cellular metabolism, as shown by the feeling of well-being exhibited by a person with pernicious anemia after the treatment for a few days with liver extract. The rapid clinical improvement is not due to a relief of the anemia. It has been suggested that liver extract restores normal pteroylglutamic acid metabolism possibly by freeing it from its conjugate form in which it normally occurs in foodstuffs. According to this concept folic acid is related to the blood lesion only. Further research may well show that other specific substances necessary for normal metabolism of nerve tissue are activated by liver extract. Liver extract thus acts as an activator of cellular metabolism<sup>44</sup> rather than as furnishing specific substances preventing or relieving pernicious anemia. This work suggests that a complex type of cellular enzyme disturbance exists in pernicious anemia. The action of liver principle in restoring normal pteroylglutamic metabolism probably constitutes only one of its therapeutic effects.

#### SUMMARY

I have tried to review and clarify steps leading to our present knowledge of pernicious anemia as a clinical and etiologic entity. The early history is most illuminating. The development of the present concept of this complicated disease is a triumph of medical research. Many great names both in clinical and research fields are associated with the advance in knowledge of pernicious anemia. Further research will almost certainly clarify problems still unsolved.

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## TWENTY YEARS OF LIVER THERAPY

By WILLIAM P. MURPHY, M.D.

A PERIOD of slightly more than twenty years has elapsed since the introduction of the use of liver for the treatment of the patient with pernicious anemia. It may be of interest and instructive to consider how well the early predictions in regard to the effects of this treatment have been substantiated and to summarize briefly the progress which has been made during this twenty year period.

The intensive and controlled study of the use of liver in the treatment of pernicious anemia which demonstrated its efficacy and assured its general acceptance was begun in the spring of 1925. The results of these studies as carried out on 45 patients were published in August of the following year.<sup>1</sup> Although diets, some including liver, had been previously tried, it remained for this study to establish liver therapy on a quantitative basis as indicated by the following quotation from this first paper: "If liver and similar food is of value, every means must be taken to get patients to eat daily as much as possible, preferably 200 Gm. or more. Failure could be attributed to taking too little of such food."

At the end of another year and after observation of the effect of therapy in 105 patients, it was possible to predict with greater confidence: "Successful therapy of pernicious anemia depends on the treatment being properly carried out for a correctly diagnosed case. With these conditions established, we believe that essentially all patients with pernicious anemia can be benefited, and usually markedly and promptly."<sup>2</sup>

Even though the method of treatment has been greatly changed and simplified since the two statements quoted above were written, the predicted beneficial effect of liver (or its extracts) has been confirmed and the advice contained in them relative to treatment has been found to be as important now with the simplified methods of treatment as it was when whole liver was used. Failure to obtain the best results possible are all too frequently the result of the use of insufficient amounts of anti-pernicious anemia substances or to their use only after irreparable damage has been done—too little, too late.

The stages in the progress of treatment from whole liver to an extract of liver for peroral and finally parenteral administration during this twenty year period are so well known that they need not be here repeated. Although several patients have been well maintained for years entirely with the use of whole liver or extracts for peroral use, the treatment of choice for the great majority of patients is with liver extracts for parenteral administration. These extracts are now fairly well standardized and their potency controlled so that the physician has access to highly potent and refined ones which produce a maximal response with a minimum of inconvenience and discomfort to the patient.

The most rapid and in most instances the most satisfactory response to treatment during relapse has been found to follow the injection of large doses of extract

supplying a high content of anti-pernicious anemia substances as elsewhere outlined<sup>3 4</sup> Thereafter maintenance treatment must be individualized, determined on the basis of its effect on the blood levels and on the neural disturbances if these are present. The amount of liver extract necessary to maintain the best possible state of health varies greatly from one patient to another. The need for adequate therapy cannot be too greatly stressed. In his Nobel lecture made after nearly ten years of experience with liver therapy, Minot<sup>5</sup> made the following statement: "The grave error in treatment is to prescribe too little liver extract or potent substitute. Where there is doubt, more rather than less should be given. It is essential that the individual receive into his body indefinitely and with regularity enough potent material for his given case." Little need be added to this statement in order to condemn an effort to standardize maintenance therapy. It has been demonstrated that the amount of liver extract needed to maintain a satisfactory state of health as determined from observation of a fairly large group of patients<sup>6</sup> is that which supplies 15 units about every three and one-half weeks. The actual intervals varied, however, in this group of patients from one to six weeks. It is obvious from this that one cannot produce the best results if the same dose of extract is given to all patients at the same interval.

The more highly concentrated and refined extracts insure the most satisfactory response with the least inconvenience and expense because of the need for less frequent injections. There is no valid argument for the use of so-called 'crude' extracts in the treatment of pernicious anemia even though disturbances resulting from sclerosis of the central nervous system are present. There is much evidence available to confirm the beneficial effect of the concentrated extracts on all of the disturbances characteristic of pernicious anemia including those due to neural damage. No evidence has been presented to show that "crude" extracts are more beneficial in any respect. Furthermore, their use necessitates greater frequency of injection and they are often distinctly more irritating with greater discomfort for the patient. The 'crude' extracts contain a greater amount of solids than do the refined which are probably inert, the content of vitamins and other substances which might add to their value has not been found to be greater and some of those used as 'crude' extracts are merely dilutions of the refined.

The superiority of parenteral extracts over other forms of therapy is in part due to the careful follow-up control of the patient, made possible by his return for injections at regular intervals. The pernicious anemia patient is subject to the same weaknesses of the flesh as are we all. As his condition improves with peroral therapy there is a great temptation to neglect treatment and the check-up visit to his physician, the result in too many instances is hematologic and neurologic relapse. The relatively frequent visits for injection have improved the physician-patient relationship so essential to the most satisfactory control. The importance of this is emphasized by Minot,<sup>5</sup> also in his Nobel lecture when he cautions: "The physician, however, must do more for his patient than prescribe a proper amount of liver, stomach, or the like, he should attend to all aspects of the case and not neglect attention to the individual's problems of thought and action."

Were it possible for all patients with pernicious anemia to receive treatment

sufficiently early in the course of their disease and in accordance with the principles demonstrated to be most effective, the cause of death would rarely be recorded as that of pernicious anemia. The causes of death have changed somewhat in respect to frequency of occurrence as the average age of the patient has increased in consequence of the liver treatment. Hypertensive cardiovascular disease and malignancy account for a rather high percentage of deaths as might be expected in a control group of comparable age.

The incidence of malignant disease in patients with pernicious anemia is not known. In the author's<sup>7</sup> series of 578 cases followed during the first twelve years of liver therapy, 29 instances of malignancy involving some part of the body were observed, an incidence of 5 per cent. During that same period malignancy of the stomach caused death in only 4 cases. Two more involving the esophagus were noted. During the twenty year period 50 instances of malignant disease have been observed. Twenty of these involved the stomach and in all but 1, still living, was the cause of death. Whether or not this indicates a higher incidence of gastric carcinoma than occurs in a group of patients of comparable age without pernicious anemia has not been determined. It is quite likely that this is not the case.

The recent synthesis of folic acid<sup>8</sup> and its demonstrated beneficial effect on the blood levels in pernicious anemia<sup>9</sup> has stimulated renewed interest in the therapy of the disease. Although this new development may be an important step toward solving several questions concerned with the mode of action of liver or its extracts and although the preliminary reports of its use in the treatment of pernicious anemia are encouraging, it must be remembered that its use is still in the experimental stage. Many of its possible effects are yet to be determined, as for example, the amount necessary to initiate a satisfactory remission during relapse, the amount needed to maintain over a period of years normal blood levels and whether or not that is possible in all patients. Its possible toxic effects are not known and its value in preventing or bringing about improvement of the neural disturbances remains to be seen. Evidence has already appeared to indicate that it does not control these and that it is not a complete substitute for liver or its extracts in the management of pernicious anemia. It is to be hoped that the medical profession will not be stampeded into the use of folic acid as a substitute for liver substances by glowing reports of its value made, particularly in the lay press, by irresponsible writers who do not have at heart the best interests of the patient with pernicious anemia.

Much more study is needed before folic acid can be accepted as a safe and effective substitute for liver and liver extracts. Efforts to side-step a definite decision in this regard by combining folic acid with liver extract merely increase the cost of treatment without definitely adding to its effectiveness. One may confidently say in the light of our present knowledge that liver will do everything that folic acid will do and more for the patient with pernicious anemia.

Finally it may be stated with confidence that results of the use of liver or its extracts in the treatment of pernicious anemia during the twenty years justify the early optimism in regard to its value.

Some of the patients included among the first group of 45 treated and more of

those who were included in the group of 105 reported the following year are alive and well insofar as their pernicious anemia is concerned. Except for the complications which have appeared as the result of increasing age and not related to the anemic state, many more of these original groups would now be living. Few of those who partook of sufficient liver to bring about a satisfactory remission have died from pernicious anemia either as the direct or indirect cause.

Not only have these persons been kept alive but they have been maintained in such good physical condition that it has been possible for them to carry on their normal occupations as housewives, merchants, teachers, lawyers, physicians, etc. The distressing, and often incapacitating, disturbances resulting from damage to the central nervous system have been controlled or completely avoided.

Before the introduction of the liver treatment, the number of deaths from pernicious anemia in the United States alone had risen to about 10,000 per year. It may, therefore, be estimated that 200,000 persons with this disease in this country alone have had their life span increased by at least ten years and that there are 100,000 persons now living with this disease who would not be except for their use of liver or its extracts.

In closing this brief resume of the experiences in the treatment of pernicious anemia with liver and its extracts during this first twenty year period one cannot better express the outlook for the future than did Minot<sup>5</sup> in the closing words of his Nobel address: "It seems to me that one may expect in the future more information to be obtained which, directly or indirectly, will follow as the result of these observations. Thus, upon the foundations laid by previous investigators, do medical art and science build a structure which will in its turn be the foundation of future knowledge."

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# PERNICIOUS ANEMIA, NUTRITIONAL MACROCYTIC ANEMIA, AND TROPICAL SPRUE

## A DISCUSSION

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THE BRILLIANT work of Minot and Murphy (1926 and 1927) on the curative action of liver in Addisonian pernicious anemia and the subsequent work of Castle and his colleagues (1929, 1930, 1931, 1936) on the mechanism of the formation of the liver principle opened up a vast field of research into the nature and the mode of action of this principle in pernicious anemia. It was a natural development to extend this field to include a study of other macrocytic megaloblastic anemias and that an explanation of the etiology of all these should be sought on the basis of Castle's theory of an extrinsic-intrinsic factor reaction leading to the formation of the liver principle. Much experimental and clinical work was carried out on these lines, but the concentration on this one aspect of these diseases and recently on the effect of folic acid in the same anemias has, by thus limiting the field, led to a neglect of the study of the general pathology and the natural history of these conditions and perhaps to a too limited view of their etiology. In pernicious anemia, nutritional macrocytic anemia and tropical sprue, to limit the discussion to the three principle macrocytic anemias, anemia is only one aspect of each disease complex and the fundamental differences between the three disease entities have been overlooked in the light of the spectacular success of treatment with liver extracts and folic acid. It was attractive to fit these three diseases, or rather the anemia in each case, into the framework of Castle's theory, according to which all three are due ultimately to a deficiency of the liver principle. Pernicious anemia thus would arise through a deficiency of the liver principle due to an absence from the stomach of the intrinsic factor, nutritional anemia to the same anemia arising from a lack of the extrinsic factor in the diet and the macrocytic anemia of sprue to a failure of absorption of the liver principle, in some cases associated with a reduction or absence of the intrinsic factor. Even had such an explanation in its simple form withstood the test of clinical trials and experimental work, it would not have explained the etiology of the pathologic change in the gastric mucosa in pernicious anemia, to which the absence of the intrinsic factor is attributed, nor that of the functional changes in the intestinal mucosa which lead to the failure of absorption in sprue. This is not to question the validity and applicability of Castle's work, his experimental results belong to the group of "obstinate facts" that have to be reckoned with, but the simple explanation of the causes of the postulated deficiency in 'the liver principle' in these diseases must be revised in view of certain experimental and clinical findings. Undoubted cases of macrocytic anemia due to a deficiency of the extrinsic factor do exist and respond to treatment with this factor (Moore et al , 1944, Watson and Castle, 1946). On the other hand

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in many areas where nutritional macrocytic anemia is endemic it has been shown that the anemia does not respond to treatment with purified liver extracts known to be active curatively in pernicious anemia even when these are given in doses equivalent to or much larger than those effective in pernicious anemia. However, in other areas cases have been shown to respond to enormous doses of purified extracts, when the question of traces of other factors possibly present in the extracts has to be considered (for review of literature see Watson and Castle, 1946). Animal experiments have also confirmed the inactivity of highly purified extracts in nutritional macrocytic anemia (Wills et al., 1937) and now modern work on the therapeutic activity of folic acid in macrocytic anemias has to be fitted into the picture.

A consideration of these findings necessitates a reorientation of our mode of approach to the study of these three diseases which does not imply a neglect of the study of the pharmacologic and physiologic action of the elusive liver factor or of that pleasantly concrete substance folic acid. But the approach should be widened to include the natural history of these conditions and a study of their general pathology. In these studies certain considerations should be borne in mind. It should be remembered that the response of the body to any one factor or deficiency varies with the past and present environmental condition, race, family, sex, climate, diet, illnesses, etc., all of which will have determined the physiologic and anatomic state of the body at any one moment of time. The presence or absence of one factor may condition the mode of action of another factor, starving animals on a rachitogenic diet fail to develop rickets. Metabolism proceeds by a series of chain reactions, this is beautifully seen in cellular metabolism where the members of the vitamin B<sub>2</sub> complex play such an important role in the chain of oxidation reduction reactions. The links in these chains may be broken at many points, but the breakdowns thus produced may result in very similar pathologic states. It should also be stressed that a single symptom complex, such as that of a macrocytic hyperchromic anemia may be a part of many different disease entities. Much confusion has arisen for example by the attempt to bring all nutritional macrocytic anemias into one etiological group or to differentiate types on the basis of the serum bilirubin without due consideration of complicating factors. With these considerations in view an attempt will be made to review the natural history and to study the pathogenesis and interrelationships of these three clinical entities. In each case the discussion will be limited to the classic type of each disease, though mention will be made to the large group of ill defined and at present not worked out conditions which have many of the signs and symptoms of the clinical entities under consideration.

#### GEOGRAPHIC DISTRIBUTION

The distribution of the three diseases varies widely. Pernicious anemia is mainly a disease of the temperate zones, the highest incidence being in the Nordic countries, the British Isles, Canada, and the northern portion of the United States of America. Nutritional macrocytic anemia in its endemic form is, broadly speaking, a disease of tropical and subtropical lands. Originally described as pernicious ane-

mia of pregnancy" in India (Balfour, 1927, McSwiney, 1927, Wills and Mehta, 1930), it was later reported from British Guiana (Giglioli, 1934), East and West Africa, (Trowell, 1943), and probably from Puerto Rico, for though Castle and co-workers (1935) have described the cases of macrocytic anemia they studied there as sprue, it is probable that British workers would consider some, at least, of their cases as nutritional macrocytic anemia. Rodriguez-Molina (1939) has described typical cases of nutritional macrocytic anemia from the same island. A similar anemia, which will be discussed later, has been reported from Macedonia by Fairley and his colleagues (Fairley et al., 1938, Foy and Kondi, 1939). Sporadic cases, particularly among pregnant women, have been reported by many workers from Europe and America, but the relation of these cases to the endemic diseases is uncertain. Tropical sprue, as its name implies, occurs mainly in the tropics. It was first described in 1759 by Hillary in the West Indies and then rediscovered in the Far East (Manson, 1879-80), and has since been recognized in many parts of the world. Further, it has long been observed that certain districts and even houses in endemic areas have a high sprue incidence (Leishman, 1945, Keelc, 1946).

#### SEASONAL INCIDENCE

Pernicious anemia is not a seasonal disease. The highest incidence of nutritional macrocytic anemia in Bombay is during the winter months and the lowest during the monsoon (Balfour, 1927). This variation may be related to humidity, temperature or changes in the small additions to the diet, which are of such importance in very deficient and monotonous diets. Marriage customs may also influence the incidence, as they give rise to a seasonal variation in the number of pregnant women in the community and thus to the number of cases of nutritional macrocytic anemia, as this is very prevalent among such women. Hare (1946) reports that in Assam the maximal incidence of anemia, including macrocytic anemia in pregnant women, is in the third quarter of the year, which is the rainy season there. At this time fresh green vegetables are not available and the home pounded rice normally eaten is replaced by milled rice. Napier (1941), in Calcutta found that the highest incidence in pregnant women occurred in the second half of the year, in which, owing to the hot weather and monsoon, less fresh vegetables and fish are taken. The onset of diarrhea in sprue is often associated with the hot weather, when in India the barometric pressure is lowest, but new cases arise at all times of the year.

#### POPULATIONS AFFECTED

A consideration of the racial and social groups who suffer from these three diseases is of considerable interest as they vary widely. Pernicious anemia appears to be par excellence a disease of people of European descent, particularly of the Nordic races, and is rare in Asiatic or Negro peoples (Friedlander, 1934). Within the racial group affected the disease is no respecter of persons, occurring in men and women of all social strata but showing a marked familial incidence. The individuals affected are characterized by or give a history in other members of the family of a fair complexion and hair and light colored eyes. The other two diseases are, however, class conscious. Endemic nutritional macrocytic anemia occurs in certain ill-

nourished populations, particularly among vegetarians (Taylor and Chhutani, 1945, Walters, 1947), the poverty-stricken poorer inhabitants of Indian cities (Wills and Mehta, 1930, Mudaliar, 1932, Napier, 1941) the East Indian laborer in Central America (Giglioli, 1934), Indian troops on active service (Marriott, 1945) or the returned Indian prisoner of war (Walters et al, 1947) Pregnant women are especially liable to develop the disease Napier (1941) in Calcutta found that in pregnant women suffering from anemia there is a significant correlation between severe anemia and poverty but that the correlation between macrocytosis and poverty, though very suggestive is not statistically significant His results also suggested a correlation between vegetarianism and macrocytic anemia, but this finding was partly invalidated by the fact of a high positive correlation between vegetarianism and high economic status in the cases studied

Tropical sprue, as originally described by Hillary (1759) in Barbados and as seen in India and the Dutch East Indies is, in contrast, not a disease of the ill-fed native resident, but of the relatively well-fed European or Anglo-Indian resident A distinction must be made here apparently between sprue as originally described and seen typically in India and the Far East, and "sprue" as seen in Puerto Rico In India, as mentioned above, the disease affects well-fed Europeans, in Puerto Rico the cases are reported (Castle et al, 1935, Rodriguez-Molina, 1939) as having subsisted for years on a very deficient diet, the intake of good biologic protein being particularly low This is not to imply that in the established syndrome the diet is anything but deficient, for in the untreated cases it most certainly is, the patients limiting their own diets, but the disease originates in well-fed individuals In this respect an outbreak of "acute" sprue that occurred among "the Chindits" in the Burma campaign is of considerable interest These men, picked British troops, existed for weeks on a ration that was only meant for use in a short emergency After a very short time, a matter of days, many of the men developed nausea and vomiting and practically all complete loathing of the ration and in a large proportion of the men the picture of advanced sprue developed in a matter of eight weeks (Keele and Bound, 1946) This history differs markedly from the usual one in a case of sprue and further work is necessary to fit these cases into the sprue picture Many other cases were also reported from East Asia Command by these and other workers

#### CLINICAL FEATURES

It is not proposed to discuss these in detail but merely to point out the striking differences in the three conditions A long experience of numerous cases of pernicious anemia, nutritional macrocytic anemia and tropical sprue as seen in India, makes it difficult to consider the three conditions as variants of the same disease entity As already mentioned however, it must be borne in mind that the same pathogenic agent produces widely differing clinical pictures in individuals whose genetic makeup varies, who live under diverse conditions of climate, diet, housing, etc, and who have suffered from different stresses and strains all their lives The disease entities may on the other hand be deceptively similar when one particular symptom, for example anemia, dominates the picture Disregarding for the moment



the atypical cases, which may perhaps be the clue to the interrelationship of this group of clinical entities, let us consider typical examples of each disease and compare them one with another

In pernicious anemia the patient usually presents as a well-covered, slightly lemon yellow colored, middle-aged or old individual of European descent, often already showing signs that the nervous system is involved. Very different is the picture in nutritional macrocytic anemia. The age of the patient varies from the late teens to relative old age, in India I never saw a case in a young child, but Giglioli (1934) reports 6 cases out of 51 cases below the age of 12 years, the youngest being 11 months old. The patient is commonly emaciated but in British Guiana, Macedonia and Calcutta, all areas where this anemia occurs in a population which has a high malaria rate, emaciation is not such a marked feature. Some degree of edema is common as in all severe anemias but it is sometimes extreme, occasionally from associated beri beri or hunger edema. As far as a dark skin will permit one to judge, the patient is not jaundiced, has clear conjunctivae and, with the exception of the prisoners of war, has no signs of associated disease of the central nervous system. In Bombay, where the nutritional anemia is extremely common, an occasional case may be frankly jaundiced but nearly always this was found to be associated with syphilis or malaria. In highly malarious areas such jaundiced cases are frequent. The sprue case, generally of European descent and frequently a middle-aged individual, is emaciated but with a distended abdomen often with visible peristalsis, the skin is greyish or a dirty yellow color. The signs and symptoms of other deficiencies such as tetany or purpuric manifestations may be present.

One sign which, together with the anemia, has been taken to indicate a close relationship between these conditions is diarrhea. This may occur in pernicious anemia, but is not a very constant finding and is often controlled by hydrochloric acid alone. In nutritional macrocytic anemia the occurrence of diarrhea is of considerable interest. In certain years in Bombay it was not a common complication though severe cases of nutritional macrocytic anemia occurred, in other years it was noted that diarrhea, often associated with a typhoid or hectic temperature which led to the isolation of the patient, was a very frequent complication. No specific organism could be isolated from the stool and the whole syndrome cleared on marmite or a crude liver extract and did not tend to relapse (Wills, unpublished). Giglioli (1934) reported diarrhea in only a few cases, whereas Napier (1941) reported a significant positive correlation between macrocytic anemia and diarrhea, an incidence of 42 per cent in 45 pregnant cases. A very similar sequence of events is seen in monkeys rendered anemic by faulty feeding, the anemia which is apparently the counterpart of the human condition might develop to an extreme degree without intestinal symptoms appearing or another time it might be associated with severe diarrhea which, with the anemia, improved immediately, as in the human cases, on treatment with active preparations of liver or yeast. In the animal cases too no specific organism could be isolated from the stools (Wills, unpublished). In returned Indian prisoners of war with a high incidence of nutritional macrocytic anemia, diarrhea was rarely complained of and was present in only 5 per cent of those requiring hospital treatment (Walters et al, 1947). In sprue,

which commonly develops insidiously but may develop suddenly, sore tongue and diarrhea dominate the clinical picture. The type of stool is characteristically bulky, greasy, frothy and pale, differing from that seen in the diarrhea complicating nutritional macrocytic anemia of men and monkeys, where it is usually more watery and neither so pale or so frothy. But atypical enteric stools may occur in the acute phases of sprue. The sore tongue, which also occurs in pernicious anemia and nutritional macrocytic anemia is a far more constant feature in sprue.

Nervous lesions other than signs of neuritis are lacking in both nutritional macrocytic anemia and tropical sprue. In Bombay in several hundred cases signs or symptoms of subacute combined degeneration were absent and Fairley (1936) reports the same absence in 450 cases of sprue seen by him personally. Ashford (1932) in a review of 3,000 cases of sprue does not mention any signs or symptoms of this complication (quoted by Fairley, 1936).

#### PATHOLOGY AND BIOCHEMICAL FINDINGS

Since the introduction of liver therapy the uncomplicated case of pernicious anemia seldom comes to postmortem, but such was not the case previously and both the older pathologists and the older literature can give a detailed account of the findings. Details of the pathology of nutritional macrocytic anemia are not available owing to the difficulty of obtaining permission for postmortems in such cases. It is also regrettable that most of the little material that is available came from cases living in areas where malaria is endemic and which were all examples of so-called "hemolytic nutritional macrocytic anemia" (Fairley, 1938), these cases differ in important respects from uncomplicated ones, which for clarity will be referred to as nonhemolytic nutritional macrocytic anemia.

At postmortem the body in a case of pernicious anemia is usually that of a well-nourished, middle-aged or elderly man or woman, the skin and sclerotics and particularly all the fatty tissues are a bright lemon yellow color and there is an excess of fat in and around the organs. In contrast in nonhemolytic nutritional macrocytic anemia and in sprue the body is usually emaciated, fat being conspicuously absent from all the organs and the characteristic lemon yellow color of pernicious anemia is also missing, all the organs are extremely pale. A further feature of these two diseases is the great reduction in the size and weight of the organs, especially the heart and liver, which is in contrast to the findings in pernicious anemia where the organ weight is not reduced and may be increased. Fairley (1930) thinks this decrease in organ weight may be of diagnostic significance. At the postmortem of a case of nonhemolytic nutritional macrocytic anemia, a male of about 23 years of age, the body weight was found to be under 6 stone, though he was of average height for an Indian and the heart weight was only 140 grams (Wills, unpublished). Mackie and Fairley (1929) report a heart weighing only 90 grams in a case of sprue that came to postmortem. In contrast are the findings in the hemolytic type of nutritional macrocytic anemia, in two incomplete postmortem examinations, on a pregnant woman and on a woman who had just been delivered respectively, (Fairley et al., 1938), the bodies were relatively well-nourished, subcutaneous fat was plentiful and of the same bright lemon yellow

color as is seen in pernicious anemia. The organs, particularly the liver and spleen were enlarged and the heart, examined only in one case, showed some fatty degeneration.

The hemopoietic organs and the blood picture are of particular interest in the three diseases. The classic picture of a panhemopoietic dystrophy characterized by a megaloblastic erythropoiesis, a similar disturbance in the myeloid series with pathologic macro-myeloid cells and a reduction in number and abnormality in type of the thrombocytes, is present in all three conditions and the general opinion is that the changes in the cells in marrow and blood are identical in the three diseases. But both in sprue (Mackie and Fairley, 1929) and in nutritional macrocytic anemia (Balfour, 1927, Mitra, 1931, Wills, unpublished) examination of the tibia may show an aplastic marrow with a curious gelatinous appearance in the shaft, though in other cases the red marrow may extend from end to end of the bone. In nutritional macrocytic anemia in monkeys the tibial bone marrow may show similar red and gelatinous changes with a megaloblastic hyperplasia (Wills and Stewart, 1935). The detailed picture in the bone marrow revealed by sternal puncture preparations varies from case to case with the severity of the anemia and with complicating factors, but the essential pathology is the same and the changes resulting from adequate treatment with liver or folic acid are also the same. But there is a remarkable difference in the blood condition which has not been adequately stressed. In true pernicious anemia in relapse there is some factor constantly present which causes an increase in the serum bilirubin which gives rise to the characteristic coloring of the skin, sclerotics and of the body fat and also to an increased output of urobilin or urobilinogen in the urine and feces. Fairley (1941) has also shown the presence of methemalbumen in the plasma, which is taken as evidence of intravascular hemolysis. In uncomplicated cases of tropical nutritional macrocytic anemia in relatively nonmalarious areas and in uncomplicated cases of sprue the findings are in marked contrast to those in pernicious anemia in relapse and in the hemolytic type of nutritional macrocytic anemia as shown in table 1.

Earlier figures, lost during the blitz, from a larger series of cases of nonhemolytic nutritional macrocytic anemia gave similar findings, the mean figure for 50 cases being 0.33 mg per 100 ml. Only 1 in 36 cases had urobilin or urobilinogen in excess in the urine. In the hemolytic type of nutritional macrocytic anemia seen in Macedonia the serum bilirubin in the cases reported by Fairley and colleagues (1938) was markedly raised, the mean figure being approximately double that of the author's series of untreated cases of pernicious anemia (see table 1). In an earlier series of 48 cases of sprue seen in Bombay by Fairley only 3 had bilirubin values above 0.6 mg per 100 ml. In a series of cases of "sprue" seen in Puerto Rico (Castle et al., 1932) the icterus index was determined in 89 individuals, in 24 it was above 6 units, which was almost as great an incidence of a raised value as in the same authors' series of cases of pernicious anemia. It is difficult to assess these figures as Puerto Rico is a malarious area. The difference in the figures for serum bilirubin and urinary urobilin or urobilinogen in the different clinical entities suggests that in untreated pernicious anemia and in the hemolytic type of nutritional macrocytic anemia there is a hemolytic factor which is absent in uncom-

plicated nutritional macrocytic anemia and sprue. The postmortem findings, though these are scanty in nutritional macrocytic anemia, are of interest in this connection.

The characteristic deposits of iron found in the liver, spleen, bone marrow and kidneys in pernicious anemia are not considered by most recent workers to be an index of increased hemolysis but rather of an inability of the blood forming organs to deal with the iron liberated by normal blood destruction (Minot and Strauss, 1943). But the iron in the kidneys is deposited within the cells of the excreting tubules, particularly in the proximal tubules, the glomeruli containing no iron pigment. Muir and Young (1941) have shown that large amounts of hemosiderin may be deposited in the cells of the tubules as a result of a hemolysis insufficient in degree to cause hemoglobinuria, and they suggest that these deposits in the kidney in pernicious anemia are due to such a hemolysis. Evidence in support of this view is the presence of methemalbumen in the plasma (Fairley, 1941). There are insufficient adequate postmortem studies to compare the hemosiderin deposits

TABLE 1 — *Serum Bilirubin Values*

Group	Sex	No	Mean mg. per 100 ml	S D	C V	Range mg per 100 ml
Normal*	M & F	100	$0.539 \pm 0.0247$	0.247	45.9	0.2-1.7
Pernicious anemia in relapse†	M & F	27	$1.059 \pm 0.1336$	0.694	65.47	0.4-2.5
Nutritional macrocytic anemia untreated‡	M & F	42	$0.516 \pm 0.0375$	0.243	47.1	0.1-1.0
Hemolytic N M A §	F pregnant	37	2.0			0.7-4.2
Tropical sprue	M & F	20	0.37			

\* Vaughan, J. M. and Haslewood, G. A. D. *Lancet* 1, 133, 1938

† Wills, L. 27 consecutive cases seen at the Royal Free Hospital (unpublished)

‡ Wills, L., and Evans, B. Forty-two cases seen in Bombay, 1937-8 (unpublished)

§ Fairley, N. H. et al. 1938. Consecutive cases of Nutritional Macrocytic Anemia (hemolytic type) in pregnant women seen in Macedonia

|| Fairley, N. H. 1930. Tropical sprue

S D = Standard deviation C V = Co-efficient of variation

in complicated nutritional macrocytic anemia and sprue with those in pernicious anemia. The size of the liver and spleen are of some interest in this connection. In pernicious anemia both organs are commonly moderately enlarged and show the characteristic deposits of hemosiderin and may show areas of extra medullary blood formation, fatty changes are very marked in the liver. In nutritional macrocytic anemia the size of the liver and spleen appears to vary with the geographic locality and the malarial infection rate. In Bombay, particularly in the male cases, these organs were rarely palpable and in the 2 postmortems the size of the organs was reduced, in one case the liver weighed only 630 grams and the spleen 120 grams, both organs gave a positive Prussian blue reaction. In some of the female patients both organs were just palpable. In Calcutta, British Guiana and Macedonia, all highly malarious areas, the findings differ markedly. Napier (1941) using pregnant cases, reports "a very marked association" between splenomegaly and severe macrocytic anemia, and in 6 out of 44 cases the spleen was below the navel.

In most of the cases with enlarged spleens the liver was also enlarged and there was a definite correlation between a raised Van den Bergh and enlargement of these organs. There was also a strong suggestion that there is some association between a positive Wassermann reaction and a macrocytic anemia. Giglioli (1934) found the spleen enlarged, generally to below the umbilicus, in 94 per cent of his cases of macrocytic anemia and associated in the majority of cases with a raised serum urobilin. There was a marked positive correlation between the spleen and parasite rate and the incidence of the anemia, which suggests, he thinks, that chronic malarial infection is 'a factor of very considerable importance' in the etiology of nutritional macrocytic anemia. In Macedonia the vast majority of the cases had grossly enlarged spleens.

In 2 cases of nutritional macrocytic anemia dying after parturition, postmortem examination (Balfour, 1927) showed very slightly enlarged spleens which, with the livers, gave a positive Prussian blue reaction. In the 2 cases of hemolytic nutritional macrocytic anemia in pregnant women examined postmortem by Fairley and colleagues the liver was very enlarged in 1 case with nutmeg changes from heart failure, and moderately enlarged in the other, both livers showed marked hypertrophy of the reticulo-endothelial cells with swollen Kupffer cells, phagocytosis and proliferation in the sinusoidal system. Malarial pigment was present in some of the Kupffer cells and hemosiderin in the liver cells, particularly in the outer zone of the lobules. The spleen was enlarged and hard in both cases (25 and 22.7 ounces respectively) and showed a hyperactive reticulo-endothelial system with some malarial pigment in the cells and a reduction in the lymphoid tissue, the hemosiderin was less than in the liver. The kidneys gave a negative Prussian blue reaction. Mitra (1931) found similar changes in material from cases in Calcutta which were probably hemolytic, but in his cases there was a fatty degeneration of the central part of the lobule with extravasation of blood.

The other systems of particular interest are the alimentary and nervous systems. In all three conditions the tongue may show characteristic changes but these may be absent and are not specific, as very similar changes occur in microcytic anemia and in pellagra. Abnormalities of the stomach and intestines may be present in all three diseases and are of fundamental importance in pernicious anemia. Many workers, particularly Castle et al., (1935) have compared the gut changes in pernicious anemia and 'sprue' with those seen in pellagra and have stressed the atrophic tongue changes and the diarrhea, and the similar effect of treatment on these states in all three conditions. The dramatic effect of folic acid on the intestinal symptoms in pernicious anemia, nutritional macrocytic anemia and sprue also suggests that the lesions of the alimentary tract are similar in all three conditions. But though this may be true of the final state of the fully developed disease, the basic pathology would appear to be different in the three entities. The classic work of Magnus (1938) and Meulengracht (1939) has shown that the fundamental change in pernicious anemia is an atrophy, possibly genetic, of the mucosa of the fundus of the stomach and it is this lesion which appears to lead, by the production of an abnormal gastric juice, to a failure in the supplies of the liver factor or factors necessary for proper hemopoiesis and for the good health of the central nervous

system Jacobson (1939) would correlate the presence of similar hemopoietic properties to those of liver in dessicated stomach and small intestine to the presence of argentaffin cells, which are markedly reduced in pernicious anemia, both in the atrophic gastric mucosa and in the intestine. The characteristic changes in the gastric mucosa seen in all true cases of pernicious anemia are absent from the gastric mucosa of monkeys suffering from nutritional macrocytic anemia (Magnus—from a study of our material—unpublished) so presumably they are also absent from the stomach in human nutritional macrocytic anemia. The presence of free hydrochloric acid in normal amounts would also suggest that the mucosa is undamaged. Suitable material for the study of possible changes in the gut in nutritional macrocytic anemia is not available but from analogy with animal material there is probably a thinning of the gut wall due to the general emaciation and little else in the small intestine. Nonspecific ulceration of the large intestine was seen in one tropical case that came to postmortem (Wills, unpublished). The importance, in the examination of the intestines, of fixation immediately after death is well illustrated in the postmortem reports on cases of sprue. In 1929 Mackie and Fairley reported changes in the small intestine which they considered "begin as an inflammation but pass on to degenerative changes" (Fairley, 1930). More recent work (Mackie and Fairley, 1934) on material fixed immediately after death has failed to reveal any pathologic change except slight congestion of the margins of the valvulae connivents and these authorities consider that the changes in the gut which result in such profound metabolic disturbances are functional and not pathologic. Hanes (1942) confirmed this absence of pathologic change, except extreme emaciation, in 4 fatal cases of sprue. Koppisch (Suarez et al., 1947) reported the postmortem findings in 16 cases of "sprue" in Puerto Rico. He found evidence, but it may have been a postmortem artifact, of chronic gastritis in all but 3 cases and moderate atrophy of the gastric mucosa in half the cases. Gastroscopic examination confirmed the presence of atrophy of the gastric mucosa (Rodriguez-Ollerios, 1938, Hernandez-Morales, 1944), but Rodriguez-Ollerios considers that this atrophy results from the disease and does not precede it, as the atrophy was only found in fully developed cases. Hernandez-Morales found that after treatment the mucosa in many cases became normal again. Koppisch also reported a definite shortening and blunting of the villi of the small intestines, with an associated increase in the number of plasma cells in the tunica propria in half the cases examined. As in nutritional macrocytic anemia in monkeys and man, inflammation and nonspecific ulceration were found in the colon of the majority of the cases.

The nervous lesions of subacute combined degeneration of the cord, so characteristic of pernicious anemia, are rarely, if ever, seen in true tropical sprue or nutritional macrocytic anemia. Peripheral neuritis may occur in all three conditions but is an inconstant finding due to an associated dietary deficiency in the vitamin B complex in the nutritional cases and probably to conditioned deficiency in the case of sprue.

Chemical examination of the gastric juice in the three conditions shows a complete and persistent histamine resistant achlorhydria associated with achylia and absence of the intrinsic factor in true pernicious anemia, in nutritional macrocytic

anemia and sprue the gastric acid varies from hyperchlorhydria to hypochlorhydria and achlorhydria, the last being more frequent in cases of sprue than in cases of nutritional macrocytic anemia. The amount of intrinsic factor present in the gastric juice of such cases is also variable, being absent in some cases of sprue (Castle et al, 1935) and in certain cases of nutritional macrocytic anemia associated with pellagra (Moore et al, 1944). It has not been possible to test the gastric juice of cases of uncomplicated tropical nutritional macrocytic anemia, as no cases of true pernicious anemia were available in Bombay for test purposes.

Various biochemical tests are now used for the diagnosis of sprue, the most important being the fat content of the stool, the fat absorption test and blood lipid curves and the oral glucose tolerance test. It is doubtful whether any of these can be considered to give specific diagnostic results as similar findings to those usually obtained in sprue cases are also found in other conditions, such as multiple vitamin B complex deficiencies, but in conjunction with a typical history and clinical picture these tests can confirm the diagnosis. In sprue the stools are typically bulky, pale and fermenting, the color being commonly pale but dark colored stools being not uncommon (Black, 1945). The stools contain an excess of fat, the greater part of which is split. Fat balance experiments show a decreased absorption and though after treatment the diarrhea may be controlled and the percentage of fat in the stools decreased, fat absorption is still defective and it may be some considerable time before it improves (Black et al, 1946, Davidson et al, 1947). In nutritional macrocytic anemia the stools frequently appear normal (Wills, unpublished, Walters et al, 1947). Fairley and colleagues (1938) give figures showing a low fat content with normal ratio of split to unsplit fat. Such values would be expected in most of the cases as poverty limits the fat intake. A dietetic survey in Bombay among the families of patients with nutritional macrocytic anemia showed that the average daily consumption per adult was 45 Gm, of which 20 Gm was animal fat (Wills and Talpade, 1930). Cook (1944) and Chandhuri (1944) have described a spruelike condition in the civil population in India associated with macrocytic anemia and diarrhea but the diarrhea was watery and not fatty in both series. Walters (1947) and Girdwood (Davidson et al, 1947) however, describe a steatorrhea in a deficiency syndrome resembling sprue in Indian soldiers. In certain of these cases the diarrhea improved with nicotinic acid and in many sulphaguanidine controlled it, suggesting an underlying infective condition (Marriott, 1945, Chandhuri and Chandhuri, 1944).

The typical flat oral glucose curves that occur in sprue are not found in the uncomplicated case of nutritional macrocytic anemia (Fairley et al, 1938) but in those cases associated with a spruelike syndrome flat curves are found in a few instances (Chandhuri and Chandhuri, 1944).

#### TREATMENT

Since the epoch making discovery of Minot and Murphy of the therapeutic activity of liver in pernicious anemia, treatment of this and other allied macrocytic anemias has involved the use of different liver extracts, with stomach preparations, and in the nutritional cases with various so-called sources of Castle's extrinsic

factor But until recently, when the discovery of the hemopoietic activity of folic acid and its conjugated forms at last gave workers a chemically pure active substance, no pure substance with similar activity was available, the various factors postulated in Castle's theory of the formation of the liver principle and the principle itself having remained elusive This fact makes the interpretation of the activity or inactivity of different so-called purified preparations difficult, as varying doses mean varying amounts of substances other than the liver principle which, when massive doses are given, may be present in large enough amounts to be active This may explain certain of the contradictory results reported in the treatment of nutritional macrocytic anemia with some of the more highly purified extracts

It is not proposed to go into the vast literature on the therapeutic use of liver and stomach extracts and of folic acid but only to deal with those aspects of this work which have a bearing on the etiology of the three conditions under consideration In the early days of crude extracts, pernicious anemia, nutritional macrocytic anemia and the macrocytic anemia of sprue all responded well to liver preparations It was originally thought that nutritional macrocytic anemia was due to a lack of Castle's 'extrinsic' factor but doubt was thrown on this explanation when it was found that relatively purified extracts, known to be potent in cases of pernicious anemia in relapse, were completely inactive in the same or larger doses in the nutritional macrocytic anemia of monkeys, though campolon, a very crude liver extract was active curatively in relatively small doses (Wills et al, 1937) This work was confirmed by the same authors (1938) in a series of cases of nutritional macrocytic anemia in Bombay, by Napier (1939) in Calcutta, though he found that large doses of the same "purified" extract as that used by Wills and co-workers was active curatively in a few cases, and by Giglioli in Central America (personal communication) Various workers in different parts of the world have shown that "purified" extracts may be active in certain types of nutritional macrocytic anemia, as for example that occurring as a complication of pellagra (Moore et al, 1944) and in enormous doses in the hemolytic type in women, particularly in pregnant women in Macedonia but not in men in the same area (Fairley et al, 1938, Foy, 1939) Cases of nutritional macrocytic anemia also respond to marmite (autolysed yeast) and other so-called good sources of the extrinsic factor when given by mouth Recently Castle and co-workers (Watson and Castle, 1946) have shown that more than one type of nutritional macrocytic anemia occurs one that responds as pernicious anemia does to the highly purified liver extracts given parenterally in normal doses, another that responds to an unknown factor, "Wills factor" as Castle calls it, present in crude liver extracts and yeast, given either orally or parenterally, but not to purified liver extracts given parenterally, and finally one that responds to purified liver extracts given parenterally when the dose is increased tenfold Such an enormous dose might contain sufficient "Wills factor" to produce a remission

In sprue the macrocytic anemia has been shown to respond to marmite by mouth, to crude liver extracts by mouth and parenterally and also in many cases to purified liver extracts parenterally A high protein diet increases the hemopoietic effect

Finally folic acid has been shown to produce remarkable hemopoietic responses



in all three diseases. In pernicious anemia folic acid, either parenterally or orally, induces in the vast majority of cases a maximal reticulocyte response followed by an immediate rise in the red and white cell counts. The dose necessary to produce this effect is 5 to 10 mg daily or a single dose of 100 mg. Jacobson (1947) by incubating folic acid with the enzyme xanthopterase, has thereby enormously enhanced the hemopoietic activity of the folic acid, and he suggests that by this means folic acid has either been converted into Castle liver principle itself or into another compound with great hemopoietic activity. It is of interest in this connection to note that both the cases of pernicious anemia treated with the incubated material showed a steady rise in the red cell count and hemoglobin percentage to normal levels, the count reaching the 50 million level and the hemoglobin a corresponding one. This is in contrast to most workers' experience with folic acid, they find that often, after an excellent initial response, it is impossible, even with increasing doses, to get or maintain the blood at really optimal levels (Wilkinson, 1947, Davidson and Girdwood, 1947, Goldsmith, 1947, Meyer, 1947). Folic acid produces in all three of the anemias under consideration an immediate sense of well-being, of the same order as that produced by an active liver extract. But again in the treatment of nutritional macrocytic anemia the blood fails to reach normal values and macrocytosis persists, this is particularly so in cases of nutritional macrocytic anemia with diarrhea and also in cases of sprue, though the general clinical improvement is remarkable (Davidson et al, 1947, Morrison and Johnston, 1947, Suarez et al, 1947). It also has a miraculous effect in controlling the diarrhea of sprue and nutritional macrocytic anemia, though analyses have shown that in spite of the steatorrhea being decreased there is no immediate alteration in fat absorption (Suarez, Spies and Suarez, 1947, Davidson et al, 1947, personal cases).

In contrast to the general dramatic improvement is the complete ineffectiveness of folic acid treatment in arresting or preventing the development of symptoms of subacute combined degeneration (Spies and Stone, 1947, Wilkinson, 1947). The significance of these findings will be reviewed in the discussion on etiology of these three diseases.

In brief it can be said that pernicious anemia, including the symptoms of subacute combined degeneration can be successfully treated and health maintained with crude or purified liver extracts given parenterally or orally and proteolysed liver extract by mouth, by different preparations of hog's stomach by mouth and by digests of beef muscle or autolysed yeast with normal human gastric juice. Folic acid and its conjugated forms (Spies et al, 1947) produces a remission, which is often suboptimal, of the hematologic symptoms, an immediate sense of well-being but no effect on the nervous symptoms, the hematologic effect is said to be enhanced by incubation with xanthopterase.

Nutritional macrocytic anemia, both the nonhemolytic and hemolytic types seen in endemic form in many tropical countries, responds to crude liver extracts parenterally or by mouth and to autolysed yeast extracts (Wills, 1938, Napier, 1939). Relapses do not take place after cessation of treatment if the diet is improved. Folic acid has, in the few cases reported, the same action as in pernicious

anemia (das Gupta and Chatterjee, 1946) \* The hemolytic type seen in Macedonia is very resistant to treatment but some cases respond to enormous doses of both crude and purified liver extracts and to very large oral doses of marmite (Fairley et al, 1938, Foy and Kondi, 1939)

The treatment of sprue has been studied by Fairley (1936) in cases largely from India and the Far East, by a group of workers in Cuba and Puerto Rico (Spies et al, 1946-47) and by the service authorities in India and the Far East where the disease was of relatively rapid onset (Leishman, 1945, Macgraith et al, 1945, Keele and Bound, 1946) All agree that for optimal improvement a high protein diet with liver extract or folic acid are required In the critically ill, blood transfusion may be necessary and in the service cases sulphaguanidine often controlled the diarrhea The high protein diet leads to an improved nutrition, as the absorption of protein does not appear to be affected

### PATHOGENESIS AND DISCUSSION

Our present knowledge of the three clinical entities described supports, in the author's opinion, the view that they are three distinct separate diseases, with essentially different natural histories and pathological pictures It is now proposed to discuss the evidence for this belief in the light of the facts set out in the previous paragraphs Authorities will not be quoted when they have already been given It is proposed to limit this discussion to the classic conditions as generally understood Pernicious anemia is a well recognized disease in which a persistent achylia gastrica is a diagnostic feature The title nutritional macrocytic anemia is limited to the disease as seen in endemic areas but will include sporadic cases occurring in other parts of the world, but to simplify the discussion the sporadic cases of "pernicious" or "macrocytic" anemia of pregnancy will not be considered, as it is felt that this group probably includes several different entities Tropical sprue is less well defined and recent experience in the services has led to the inclusion under this title of certain relatively acute conditions closely resembling sprue, but not yet sufficiently worked out to be definitely included under the title of "tropical sprue," which for the purposes of this discussion will be limited to the classic disease as described by workers from Hillary in 1759 to Fairley in 1938 The service cases and "sprue" as seen in Puerto Rico will be discussed in relation to the classic picture

A consideration of the geographic, ethnic, and social distribution of these three diseases leads to the conclusion that they are three separate entities Pernicious anemia is a familial disease of persons of European descent, with certain very definite genetic characteristics, the distribution of the disease corresponding to that of the racial groups affected, chiefly fair and Nordic peoples Individuals of all classes are affected In marked contrast is the distribution of nutritional macrocytic anemia This disease occurs mainly in tropical and subtropical lands, but the

\* Since the writing of this paper, Kemp (Lancet 2 351, 1947) has reported 3 cases of nutritional macrocytic anemia who showed remarkable improvement with folic acid but were not studied long enough to show whether the blood level would have reached completely normal values and whether the macrocytosis, still present with red cell counts at the 4 million level, would have disappeared

distribution is associated with poverty, a low calory, largely or entirely vegetarian diet, with pregnancy and lactation and also with certain diseases such as syphilis and particularly chronic malaria, which result in a hypertrophied reticulo-endothelial system. It also occurred in vegetarian Indian troops under the stress of service conditions and in Indian prisoners of war. An anemia considered the animal counterpart of the human disease can be produced in monkeys by feeding with a diet based on one in common use among sufferers from this disease. Pregnancy and lactation are known to increase the maternal requirements and these conditions will convert a latent deficiency into an overt one. The relation of chronic malaria to this anemia will be discussed later. These findings strongly suggest a direct nutritional origin, in other words that this anemia is an unconditioned deficiency state. The fact that this anemic syndrome is often associated with other symptoms including diarrhea, often watery but sometimes fatty, referable to a vitamin B<sub>2</sub> deficiency supports the view that the anemia is a deficiency state. The geographic, ethnic, and social distribution of tropical sprue presents a much more difficult problem. Classic sprue has generally a gradual onset and is associated with residence in a warm climate but affects Europeans rather than pure Indians or Negroes. The nature of the illness, which is generally afebrile throughout, throws little light on the etiology but the result of treatment suggests a deficiency state. Though this certainly exists in the fully developed syndrome there is little evidence for a nutritional origin for the altered intestinal absorption which conditions the deficiency. A more detailed examination of the distribution of the condition, the heavy incidence in certain areas in the large endemic zones and even in certain houses, combined with the fact that very many sprue patients come from the ranks of the well-fed, suggests a possible infective agent as the primary cause but this is purely conjectural. A consideration of the outbreaks of acute sprue in the service is of interest in this connection, though further study is necessary before these can be definitely considered the same clinical entity as classic 'tropical' sprue. Sprue in service personnel showed the same concentration in certain districts and sometimes in certain camps, explosive outbreaks which assumed epidemic proportions in certain areas also occurred. Leishman (1945) reports from Chittagong that nine separate units were affected, some with a 50 per cent attack rate and one R A F unit had 10 per cent of its personnel down with diarrhea three weeks after its arrival in India, which diarrhea rapidly turned to the sprue syndrome. These findings are very strongly suggestive of an infective origin for this type of sprue.

A study of the pathologic and biochemical findings in these diseases supports this idea of their essential individuality. The pertinent findings in pernicious anemia are the atrophic changes in the fundus and cardia of the stomach, with its associated achylia gastrica, the increased plasma bilirubin, the presence of methemalbumen in the plasma, the distribution of iron in the tissues and other evidence of a hemolytic factor in the anemia and the changes in the central nervous system characteristic of subacute combined degeneration of the cord. The atrophy of the gastric mucosa would appear to be the basic defect, it involves all the coats of the stomach wall and results in a complete histamine resistant achlorhydria and an associated achylia. There is no evidence of preceding inflammatory processes and

Magnus (1938) thinks the evidence points to the change being the final stage of an atrophic process, the cause of which is unknown but might be 'the end result of some endocrine or nutritional deficiency or might even be congenital in origin'. The evidence for the genetic factor is the familial and racial incidence and the lack of any indications of an infective, nutritional or endocrine origin. Idiopathic hypochromic anemia, an iron deficiency anemia associated with achylia gastrica also occurs in families subject to pernicious anemia (Wintrobe and Beebe, 1933). In sprue an atrophy of the gastric mucosa has been reported by the Puerto Rico workers but the evidence points to it being secondary to the disease, as it occurs only in the fully developed syndrome. Mackie and Fairley, from a study of specially fixed material, report a normal mucosa. In the nutritional macrocytic anemia of monkeys there is no significant change in the gastric mucosa and presumably the mucosa is normal in the corresponding human conditions. Test meal findings confirm the essential normality of the gastric mucosa in most cases of sprue and nutritional macrocytic anemia. The defect in the gastric mucosa seen in pernicious anemia according to Castle's well known theory produces a lack of his intrinsic factor, a substance with enzymic properties, and it is this deficiency that leads to the failure of the formation of the liver principle. Castle's work has shown the mode of action of this intrinsic factor, but neither it nor the extrinsic factor have been isolated, any more than the liver principle itself.

This defect in the gastric secretion, with its interference with the formation of the liver principle and possibly with another principle essential for the proper functioning of the nervous system, appears to be the basic lesion in pernicious anemia, but unless we accept the view that a certain variable time is necessary for the postulated genetic factor to bring about this gastric atrophy it is necessary to look further for a factor producing this atrophy, from the evidence it seems that there is little to suggest an inflammatory one. This same time factor in the development of the symptoms of pernicious anemia appears to operate in those cases which develop the disease after total gastrectomy, a period as long as ten to fifteen years occurring between the time of operation and the time of development of symptoms (Meyer et al., 1941). This time lag is also unexplained. In this connection Rhoads's (1933) experiments on the production of a syndrome resembling pernicious anemia in hogs by feeding modified black tongue diets are of interest. The deficiency not only produced tongue changes, a macrocytic anemia and nerve lesions, but a histamine resistant achlorhydria, this syndrome, though it resembled that of pernicious anemia, differs from it in that the gastric changes are reversible and the condition could be cured, whereas in true pernicious anemia replacement therapy is always necessary, the primary lesion being irreversible.

Another experiment of interest in this connection is that of Petri (1944) and co-workers, these authors have shown in experiments on dogs and swine that total gastrectomy produces signs of pellagra, due apparently to interference with absorption of nicotinic acid. Furthermore the livers from such pigs were ineffective in the treatment of pernicious anemia. However, nicotinic acid by the parenteral route compensated in these animals for the absence of gastric secretion, the livers from gastrectomized pigs receiving parenteral nicotinic acid being fully effective.

in pernicious anemia in relapse. This experiment indicates one of the factors that may influence the formation of the liver principle and may explain the pathogenesis of those macrocytic anemias which respond to nicotinic acid (Cook, 1944).

The hemolytic process seen in pernicious anemia is of great importance both in a consideration of the essential pathology of the disease and in considering its relationship to nutritional macrocytic anemia and sprue. There is little to explain the increased serum bilirubin and other evidence of increased red cell destruction except that it ceases under appropriate treatment with liver, hog's stomach or folic acid and might, therefore, be thought to be due to the nature of the macrocytes. There is, however, no increased fragility of the red cells and no hemolysis has been demonstrated in the blood stream but there is evidence of active phagocytosis of the red cells in the reticulo-endothelial cells of the bone marrow, liver and spleen, which might account for some of the increased bilirubin in the circulation. But the presence of methemalbuminemia and a positive Schumm test would suggest some additional intravascular hemolysis (Fairley, 1941). With certain exceptions already mentioned, there is no evidence of hemolysis in either nutritional macrocytic anemia or sprue and as the megaloblastic reaction is generally considered identical in all these anemias, it is unlikely that the hemolysis in pernicious anemia is due entirely to the nature of the cell. It would appear more likely that it is due to an association of an overactive reticulo-endothelial system with abnormal cells, though the cause of this hypothetical increased activity is unknown. The evidence of increased hemolysis seen in cases of nutritional macrocytic anemia occurring in areas of endemic malaria supports this view.

Tropical sprue is a dramatic disease in which spectacular pathologic changes in the tissues might be expected but postmortem examinations have failed to reveal any anatomic changes except those in the bone marrow and those of extreme inanition, the loss of fat and shrunken organs. There are no lesions of the nervous system as in pernicious anemia. Histologic examination of material from the gastrointestinal tract fixed immediately after death has shown essentially normal structures. The changes in the gut are functional and not anatomic. In a brilliant review of recent work Stannus (1942) has marshalled the evidence for a failure of phosphorylation of fatty acids, glycerol and glucose being the basic lesion, the point of functional breakdown. There is much evidence in support of this view. Maeraith and colleagues (1945) have shown in cases of spruelike conditions that in the active phase the absorption of glucose is grossly impaired though that of fructose is not, suggesting that there is an impairment of phosphorylation of glucose, although the diffusion of sugars across the membrane is unaffected. Leishman (1945) suggests that the vitamins riboflavin and nicotinic acid are concerned in this process of phosphorylation, in their phosphorylated forms as co-enzyme I and yellow oxidase they take part in cellular metabolism, acting as H acceptors or rejectors and Leishman thinks it is possible that they may catalyse the process of phosphorylation. It is possible that some other member of the vitamin B<sub>2</sub> complex takes part in this process. But if the failure in phosphorylation is due to deficiency of any B vitamin how does this deficiency arise in well-fed people and why did it not occur among prisoners of war in Japanese hands, who suffered so badly from

deficiencies of these vitamins Leishman points out in this connection that the B vitamins are synthesized in very appreciable amounts in the gut, and that a change of diet or dysentery might alter the balance of the intestinal flora and hence of vitamin synthesis Work along these lines might be illuminating

Pathologic and biochemical observations throw little light on the essential pathology in nutritional macrocytic anemia As in pernicious anemia and sprue the hemopoietic organs show the changes characteristic of a macrocytic anemia but both in nutritional macrocytic anemia and in sprue the bone marrow from the tibia (the bone examined) may show gelatinous changes as well as the extension of the red marrow characteristic of pernicious anemia Beyond these changes and those due to inanition there are no obvious pathologic lesions Magnus has shown the presence of a normal gastric mucosa in material from monkeys suffering from nutritional macrocytic anemia In man the gastric acidity and the blood sugar levels after oral glucose fall within normal limits There is no evidence of increased hemolysis, except in areas with a high incidence of malaria Fairley and Foy have given detailed accounts of this type in which the increased serum bilirubin values, a positive Schumm test, the increased urobilin output, the yellow color of skin and body fat are all indications of an increased hemolysis The spleen is enormously increased in size, frequently the liver also and postmortem material has shown a hyperactive reticulo-endothelial system Fairley sums up the picture thus "A reticulo-endothelial system irritated, activated and hypertrophied as a result of repeated malarial infections, phagocytoses these non-parasitized, abnormal corpuscles in considerable numbers, producing a haemolytic anaemia " The corpuscles are abnormal macrocytes, the result apparently of a marrow rendered megaloblastic by a deficiency of the same nature as that operation in uncomplicated nutritional macrocytic anemia

It has been suggested in the preceding paragraphs that these three diseases all exhibiting an apparently identical panhemopoietic dystrophy are distinct clinical entities It can be postulated that this dyshemopoietic anemia probably results from the breakdown of some intracellular enzyme system in which the liver principle plays an important part The relation of folic acid to this system still awaits solution but the fact that it has such remarkable, if limited, hemopoietic activity in all three conditions, as well as a dramatic effect on the well-being of the patient and on diarrhea if present, shows that it plays an all important role in rectifying the faulty cellular metabolism The liver principle is active curatively in pernicious anemia but does not seem to be the missing factor in nutritional macrocytic anemia as it has been shown to be inactive in this anemia in man and monkeys in doses equivalent to or greater than those giving maximal responses in cases of pernicious anemia with similar initial blood levels That this factor, ' Wills' factor ' as Castle calls it, is not Castle's extrinsic factor follows from the fact that the ' liver principle ' in purified preparations is inactive in nutritional macrocytic anemia Recent work on the relationship of folic acid to vitamin M deficiency in monkeys (Day, et al , 1945 and 1946, Wilson, et al , 1946) suggests that possibly folic acid is the missing factor It is possible that ' Wills' factor ' is an activator or co-enzyme in an enzyme system in which the liver principle plays the important part

Other factors may also be concerned and a deficiency in any one of these might produce a similar failure in the function of the liver principle

Finally it is interesting to consider these three entities from the point of view of preventive medicine. If a genetic defect is the ultimate cause of pernicious anemia then only selective breeding, at present a Utopian and risky measure, can eradicate it. If, as seems highly probable, nutritional macrocytic anemia is a deficiency disease due to a lack in the diet of some factor associated with good biologic protein, then improved economic conditions with the improved diet that always goes with them should eradicate the disease, except in those cases where religion limits the diet when only a new revelation can assist. But the problem of sprue awaits further work, as until we know the nature of the original cause of the intestinal breakdown it is impossible to take preventive measures.

### SUMMARY

The following tentative conclusions as to the relationship of pernicious anemia, nutritional macrocytic anemia and tropical sprue to one another and their pathogenesis are drawn from a study of the literature and from unpublished work

- 1 That these three clinical conditions are three distinct entities possessing a common characteristic in the presence of a panhemopoietic dystrophy characterized by a megaloblastic erythropoiesis and corresponding changes in the myeloid cells and platelets

- 2 That this panhemopoietic dystrophy possibly results from the breakdown of an intracellular enzyme system but that the deficiencies causing the breakdown differ, in pernicious anemia the liver principle is apparently at fault, in endemic nutritional macrocytic anemia another unidentified factor is missing, in sprue either or both may be at fault

- 3 Folic acid is active therapeutically in all three diseases, but in all it generally fails to restore completely normal blood levels

- 4 Pernicious anemia is probably due to a genetic defect which produces an atrophy of the gastric mucosa. As a consequence of this interference with gastric function there is a failure in the formation or absorption of the liver factor and possibly of another neurotrophic factor, which failure results in the development of a macrocytic megaloblastic anemia and the characteristic changes in the nervous system. Indefinite replacement therapy is necessary as the changes in the gastric mucosa are irreversible. The cause of the increased hemolysis is unknown

- 5 Endemic nutritional macrocytic anemia is an unconditioned food deficiency, the deficiency being in a factor other than the liver principle, possibly a co-enzyme present in or associated with good biologic protein and the vitamin B<sub>2</sub> complex. There are no characteristic pathologic changes except those of the hemopoietic organs which are not specific to the disease. A hemolytic type of the disease occurs in areas of high malarial incidence. After successful treatment the disease does not relapse if the diet is satisfactory. Pregnant women are particularly liable to develop the disease

- 6 Tropical sprue is due to a functional disorder of the intestine, possibly primarily a failure in phosphorylation of fatty acids, glycerol and glucose. Diarrhea

with characteristic stools and a macrocytic anemia are characteristic findings. The macrocytic anemia may be due to a failure in absorption of one or more essential hemopoietic factors or to a lack of Castle's intrinsic factor. The cause of the functional breakdown is unknown. Treatment is with a high protein diet and liver extracts. Relapses are common.

My thanks are due to my colleagues for carrying on my work while I wrote this paper.

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THE RELATION OF THERAPY IN PERNICIOUS ANEMIA TO CHANGES  
IN THE NERVOUS SYSTEM EARLY AND LATE RESULTS IN A  
SERIES OF CASES OBSERVED FOR PERIODS OF NOT LESS  
THAN TEN YEARS, AND EARLY RESULTS OF  
TREATMENT WITH FOLIC ACID

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THE FREQUENT occurrence of nervous system involvement in pernicious anemia, the characteristic localization of the lesions in the spinal cord, and the extension of the process before specific treatment became available, suggested to early observers a common cause of, or a cause and effect relationship between, the hematologic and neurologic manifestations of this disease. However, the absence of combined system degeneration or even peripheral neuropathy in many cases of pernicious anemia and the complete dissociation of the severity of the neurologic and hematologic features are not satisfactorily explained by these concepts. With the general acceptance of Castle's hypothesis of a conditioned metabolic deficiency as the basic mechanism responsible for the pernicious anemia syndrome the view was often expressed that the changes in the hematopoietic and nervous systems resulted from distinct deficiencies which, in turn, probably depended upon a primary defect in gastric function. Evidence for the existence of such separate deficiencies was difficult to obtain because of the lack of exact information pertaining to any of the metabolic factors involved.

When effective treatment of pernicious anemia was introduced by Minot and Murphy, the importance of evaluating the new therapy in the control of combined system degeneration was at once recognized. Over the past two decades many clinical reports bearing on this problem have been published, and an extensive review of the literature is not pertinent to the present communication. The diversity of results and conflict of opinions which characterized the earlier experiences with liver and stomach therapy have been largely explained as due to differences in the conditions of observation and in the amount and potency of the medications employed and the modes of their administration. It is now the consensus of most observers that with individualized optimal therapy the progress of disease of the spinal cord can be arrested in every patient with pernicious anemia unless serious complications are present, that the probability of improvement of the neurologic status and the extent of the benefit which may be expected are inversely related to the duration of the process at the time of institution of therapy, that the period during which improvement may be anticipated is largely limited to the first few months after commencement of intensive treatment.

The regular administration by the intramuscular route of potent liver extract in doses adjusted to the needs of the individual, controlled by periodic clinical and hematologic evaluation, is almost a guarantee against further nervous system

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damage. Such an ideal therapeutic regimen, however, is not employed in the actual management of many cases of pernicious anemia. Particularly is this true of those patients who live at a distance from their physicians, those who change physicians, and those who are seriously influenced by economic considerations. It therefore becomes important to evaluate the results of therapy over a long period of time in a series of cases presenting varied manifestations of the disease and differing with respect to type and amount of therapy received. It is well recognized that patients with previously active neuropathies who discontinue treatment, or those whose therapy becomes so inadequate as to permit development of pronounced anemia, almost invariably suffer reactivation of their neurologic process. It is also true, although less commonly observed, that patients without previous evidence of nervous system involvement may acquire neurologic manifestations after omission or gross inadequacy of anti-pernicious anemia therapy. It may not be so easy, however, to demonstrate a close relationship in all cases between the progress of disease of the nervous system and continued or recurrent suboptimal therapy, as evidenced by relatively slight disturbances of erythrocyte values.

The 70 patients comprising the first series to be reported have all been under observation at the Simpson Memorial Institute for ten years or longer. When seen initially they were in hematologic relapse and had received no effective antianemia therapy for at least several months prior to their first examination. Fifty-eight of the patients were previously undiagnosed and untreated. The diagnosis was established by the clinical and hematologic features characteristic of pernicious anemia, by the absence of other demonstrable conditions associated with macrocytic anemia, by the invariable presence of histamine refractory achlorhydria, and by the therapeutic response in every instance to the administration of potent anti-pernicious anemia medication. The neurologic status was evaluated and the cases were classified on the basis of objective as well as subjective evidence of involvement of the peripheral nerves and posterior columns, or of the posterior and lateral columns, of the spinal cord. Although the distinction between evidence of peripheral neuropathy, posterior column disease only, and combined system degeneration is useful as an indication of the severity of the process and in the interpretation of therapeutic results, such a separation is by no means an exact one. The extent of the process in each patient was graded primarily on the basis of peripheral nerve and posterior column involvement as one plus to four plus, indicating, in order of increasing severity: 1 plus, diminution of vibratory sense and altered reflexes in the lower extremities with paresthesia, but without significant disability; 2 plus, loss of vibratory sense and impairment of sense of motion and position in the distal portions of the lower extremities, with mild ataxia; 3 plus, complete loss of vibratory sense in the lower extremities with moderate to severe ataxia; 4 plus, ataxic or spastic paraplegia with inability to stand unassisted, sometimes associated with sphincter disturbances. Evidences of improvement were likewise graded arbitrarily on a plus basis, according to which 4 plus signifies complete disappearance of all subjective and objective manifestations. Evaluation of degrees of improvement less than complete recovery is dependent upon the

extent of the pre-existing disease and so cannot be defined in terms applicable to all patients. In general, improvement of 3 plus indicates freedom from serious disability with persistence of some paresthesia, and with slight to moderate ataxia in patients who had had the more severe forms of neural involvement. Much of the improvement of these patients may be attributed to recovery from peripheral neuropathy, with education of new muscle groups and adaptation to altered proprioceptive pathways playing important parts. However, the greatly superior results of therapy in patients, often with extensive disease, whose neurologic manifestations were of short duration, suggests that in the early stages of spinal cord involvement, nerve recovery may occur.<sup>1</sup>

The demonstration of unequivocal peripheral nerve changes in pernicious anemia<sup>2-5</sup> explains the transient and fluctuating paresthesias which occur so frequently in this condition. Peripheral neuritis is probably always present during the active periods of combined system disease and it may occur in the absence of convincing evidence of spinal cord involvement. Patients whose sole neurologic complaint was variable paresthesia or tenderness in the extremities without demonstrable deep sensory disturbances were not considered to suffer from significant disease of the nervous system. Evidence of some degree of cerebral involvement was fairly common among the members of this series. Yet the multiplicity of factors which may have contributed to the production of mental changes in these patients, including anemia, malnutrition, and degenerative vascular disease, and the difficulties involved in comparative measurements on a group of advancing age, render the precise consideration of the mental status of doubtful significance in the evaluation of long-term therapeutic results.

Several types of therapy were employed in the initial management of these patients. Some of them were among the first cases of pernicious anemia to receive the benefits of the liver diet. A considerable number were seen before parenteral liver extracts became available. During the years there has been a tendency to substitute refined and concentrated preparations of liver extract for the cruder less potent parenteral extracts and for oral products. Nevertheless, a number of patients, largely for reasons of personal choice, have continued to take oral liver extract or desiccated stomach or the cruder parenteral liver extracts. It thus becomes possible to compare the effects of different kinds of therapy with respect both to initial responses and to the neurologic status after a long period of time. Moreover, the information afforded by this analysis may be used in the evaluation of results obtained with new types of antianemia medication. In this connection, a small series of patients treated with folic acid will be reported with particular reference to changes in the nervous system.

Of the 70 cases under observation for ten years or longer, there were 45 males and 25 females, giving percentages, respectively, of 64.3 and 35.7. This is a somewhat higher proportion of males than is found in our entire series of over 1,000 cases of pernicious anemia seen at the Simpson Memorial Institute, in which the percentage of males is approximately 55. The average age of the patients at the time of diagnosis of their disease was 53.8 years, and the age distribution for the two sexes was approximately the same. The youngest member of the series was 35 and the oldest

was 68. The lower age range of these patients as compared to that generally reported for pernicious anemia is, of course, explained by the fact that they were all followed for at least ten years after the diagnosis was made. The average length of the observation period in the case of the males was 13.4 years and for the females was 13.3 years.

Objective manifestations of disease of the nervous system were present in 67.1 per cent of the patients in this group. Serious disability on a neurogenic basis was present in 15.7 per cent, but only 4 patients, or 5.7 per cent, were unable to stand or walk without assistance. It should be pointed out that the low incidence of extremely severe central nervous system disease in this series may be accounted for by the fact that the commonest causes of death in pernicious anemia are the complications of spinal cord involvement.<sup>6</sup> It is of interest, however, that, in some cases, such fatal complications may be prevented for an apparently indefinite period, even though serious disability has been present for a relatively long time before treatment is instituted. Although the incidence of neuropathy is approximately the

TABLE 1—*Extent of Neurologic Manifestations when First Seen*

	Number of cases		
	Males	Females	Total, both sexes
None (0)	14	9	23
Slight (+)	10	1	11
Moderate (++)	17	8	25
Severe (+++)	3	4	7
Very severe (++++)	1	3	4
Total	45	25	70

same for the men and women of this series, the latter tended to have manifestations of more severe involvement when first seen (table 1). Nevertheless, the differences are not sufficiently great and the number of cases is too small to warrant separate consideration of the sexes with respect to the long term course of their disease.

The changes in the neurologic status may be correlated with the duration of symptoms referable to the nervous system before institution of therapy (table 2) and with the type of treatment given (table 3). Of special significance for the purposes of this study is the correlation of the long term results with the adequacy of therapy (table 4).

The better outlook for improvement in neural manifestations when these are of short duration was first pointed out by Ungley and Suzman in 1929,<sup>7</sup> has been noted by numerous observers, and recently was re-emphasized by Rundles.<sup>8</sup> The results of treatment of the patients in this series fully support this view, but in addition they indicate that even when symptoms have been present for longer than twelve months a considerable degree of improvement will occur in most cases (table 2).

Improvement in the neurologic status was essentially limited, in all cases, to the first year of treatment, and in fact most functional recovery took place during the

TABLE 2 — *The Maximum Extent of Clinical Improvement in Neurologic Manifestations During Period of Adequate Therapy Related to the Duration of Neurologic Symptoms Before Treatment Was Begun*

Degree of improvement	Number of cases					
	Symptoms present for less than 3 months		Symptoms present for 3 to 12 months		Symptoms present for longer than 12 months	
	Extent of neuropathy					
	+ to ++	+++ to +++++	+ to ++	+++ to +++++	+ to ++	+++ to +++++
None(o)	0	0	1	0	1	0
Slight(+)	0	1	0	1	1	0
Moderate(++)	0	0	4	3	4	6
Marked(+++)	11	2	6	0	2	0
Complete recovery(++++)	3	0	1	0	0	0

TABLE 3 — *The Maximum Extent of Clinical Improvement in Neurologic Manifestations Related to the Type of Therapy Employed During Period in Which Improvement Occurred*

Degree of improvement	Number of cases							
	Desiccated stomach		Oral whole liver and liver extract		Parenteral crude liver extract		Parenteral refined liver extract	
	Extent of neuropathy							
	+ to ++	+++ to +++++	+ to ++	+++ to +++++	+ to ++	+++ to +++++	+ to ++	+++ to +++++
None (o)	1	0	0	0	1	0	0	0
Slight (+)	0	1	1	0	1	0	0	0
Moderate (++)	4	2	1	2	4	4	0	0
Marked (+++)	5	0	4	0	9	2	2	0
Complete recovery (++++)	1	0	1	0	1	0	0	0

TABLE 4 — *The Neurologic Status After not Less than Ten Years of Observation and Therapy The Long Term Results Related to the Initial Severity of the Neuropathy and the Adequacy of Treatment as Measured by the Maintenance of Normal Hematologic Values*

Extent of neuropathy	Number of cases																	
	Optimal therapy						Suboptimal therapy without definite relapses						Clinical and hematologic relapses					
	P D	0	+	++	++ +	++ ++	P D	0	+	++	++ +	++ ++	P D	0	+	++	++ +	++ ++
None	0	10	—	—	—	—	0	6	—	—	—	—	4	7	—	—	—	—
Slight and Moderate (+ to ++)	0	1	1	5	8	1	0	1	1	1	4	1	1	1	0	2	3	1
Severe and very severe (+++ to +++++)	0	0	1	7	2	0	0	0	0	1	0	0	0	0	0	0	0	0

P D signifies neurologic manifestations progressed in severity or developed during observation

0 signifies neurologic status remained essentially unchanged

— signifies absence of neuropathy, hence, no room for improvement in the disease process

first six months. The results are about equal for the different types of therapy employed, including desiccated stomach, whole cooked liver or oral liver extract, and parenteral crude liver extract, usually given intravenously (table 3). Because the more refined and concentrated liver extracts were not available when most of the patients were first seen, only 2 cases treated initially with such preparations are included in this series. However, for the past decade, refined liver extract given intramuscularly has been employed in the management of most of our new cases of pernicious anemia, and the results have been fully equal to those obtained with oral preparations and parenterally administered crude extracts. Some of these cases have been included in previous reports.<sup>8,9</sup> No patients who received optimal therapy, regardless of type, suffered exacerbation of their neurologic manifestations.

In this series, there was no apparent difference over a long period of time, with respect to changes in the neurologic status, between those patients who received the recommended amount of therapy and whose blood values were consistently within normal limits, and those in whom treatment was irregular or was inadequate as judged by variations in erythrocyte count or morphology (table 4). In neither group was development of nervous system disease observed in patients who presented no manifestations of neurologic involvement when therapy was first instituted. The incidence and degree of improvement was about the same in the two groups. However, these observations require comment, and the conclusion that irregular or suboptimal therapy provides a safeguard against development of nervous system disease is not justifiable. In the first place, all of the patients received intensive initial therapy with apparent complete arrest of spinal cord degeneration. In the second, the fact that these patients returned frequently over a period of many years is evidence that they were cognizant of the importance of adequate follow-up examination and treatment, even though they at times neglected it. Recurrence of paresthesia or of mild symptoms of anemia was a warning to them to resume active therapy. In the third place, the severity of the neurologic process in the two groups is not comparable. Of the 11 patients with evidences of extensive spinal cord involvement, 10 are included in the optimal therapy group, justifying the inference that irregular or inadequate treatment seriously affects the chances for long time survival of patients with severe nervous system disease. On the other hand, it is worthy of note that no patients in the inadequately treated group with the milder degrees of involvement showed more than transient exacerbations of their neurologic manifestations.

The patients in this series who suffered definite hematologic relapse, as indicated by an erythrocyte count of less than 3,000,000 per cu. mm. with macrocytosis did not fare as badly as might have been expected. Eleven of this group were free of evidence of neural involvement when first seen, and only 4 of these developed neurologic manifestations during subsequent relapses. In each instance the lesion was classified as moderately severe (++) and was arrested with good functional improvement when intensive therapy was resumed. Of 8 patients presenting symptoms and signs of mild or moderate degree, only one suffered irreversible progression of spinal cord damage during hematologic relapse. Here also, it should be

emphasized that the relapses suffered by these patients were generally of short duration, that the series includes only those patients who were willing and able to return, and that no patient with pre-existing severe central nervous system disease who suffered hematologic relapse has been followed for as long as ten years

### EXPERIENCES WITH FOLIC ACID

Since January, 1946, 15 patients with pernicious anemia have been treated with synthetic folic acid (pteroylglutamic acid) for sufficiently long periods to permit an evaluation of the early therapeutic results obtained with this material and a comparison of the results with those secured with other forms of treatment. Nine members of the group were males, 6, or 40 per cent, were without evidence of neuropathy, 5 had previously been under treatment for pernicious anemia, but only 1 had normal blood values at the time folic acid treatment was begun. The 6 patients without nervous system disease have all maintained normal blood values for one year or longer, while receiving 5 mg of folic acid by mouth daily, and none have developed neurologic manifestations \*

### REPORT OF CASES

One patient (H W) a man of 64, had paresthesias of the extremities, diminished knee and ankle jerks and impaired vibratory sense in the lower extremities when folic acid, 10 mg orally each day, was started during hematologic relapse. Within three months, coincident with restoration of blood values to normal, paresthesias had disappeared, and the patient had no complaints. A woman (T K), aged 69, had evidences of presumptive peripheral nerve and posterior column involvement with slight ataxia and impairment of sense of motion and position when the diagnosis of pernicious anemia was first made during hematologic relapse. On folic acid, 10 mg daily by mouth, there was significant functional and symptomatic improvement of moderate degree observed over a period of eight months. A man (C N), aged 76, had moderate involvement of the nervous system when first seen in 1938. He was treated with refined liver extract by intramuscular injection with marked (+++) improvement in his neurologic status. In April, 1946, his blood values were slightly abnormal, presumably due to too long intervals between treatments, and therapy was changed to folic acid, 10 mg daily by mouth, later reduced to 5 mg. There was no reactivation of the neurologic process at the time of institution of folic acid therapy, and none has occurred over a period of fourteen months. A woman (E C), 70 years old, was found to have pernicious anemia in 1938, with a moderately severe neural lesion. While under treatment with desiccated stomach, there was marked (+++) improvement in the neurologic manifestations. In May, 1946, treatment was changed to folic acid, 10 mg orally each day. After six months, there was no exacerbation of the neurologic process, but the erythrocyte level had declined to 3,600,000 per cu mm with a mean corpuscular volume of 105 cubic microns. Desiccated stomach, 20 Gm daily, was substituted for the folic acid and the blood values were rapidly restored to normal.

The 4 remaining patients with pernicious anemia in our folic acid treated series may be said to have had unsatisfactory results with respect to their neurologic status. In 2 of these, the adverse changes were slight and may have been equivocal, or the dosage, for these individuals, may have been too small. A physician (W F) was first found to have pernicious anemia in April 1946 and was treated initially with folic acid, 15 mg daily by intramuscular injection. His erythrocyte count was 2,600,000 per cu mm, and the maximum reticulocyte percentage, reached on the eighth day, was 15.6. He had troublesome paresthesia, especially in the toes, mild ataxia, impaired vibratory sense distal to the mid-tibiae, and swaying in the Romberg position. After one month on the parenteral 15 mg dosage, the patient felt much stronger, his appetite had improved, and he had gained weight, but there was no change in his neurogenic symp-

\* Since this report was submitted, one of the patients developed severe paraplegia while receiving 10 mg of folic acid daily.



toms The erythrocyte count was 4,300,000 per cu mm and slight macrocytosis was still present At this time the dosage of folic acid was reduced to 5 mg orally each day Two months later the neurologic manifestations and the blood values were unchanged He was then given refined liver extract, 15 units intramuscularly, every three weeks, together with the daily oral dose of folic acid, 5 mg All neurogenic symptoms, except occasional slight tingling in the toes, disappeared within two months time, and the blood values have been entirely normal for one year A woman (B C), aged 51, was first seen and diagnosed as pernicious anemia in September, 1946 Anemia was minimal, the erythrocyte count being 3,900,000, but characteristic morphologic changes were present, and achlorhydria persisted after histamine injection There were manifestations of active, moderately severe, neurologic disease, chiefly paresthesia, ataxia, and deep sensory disturbances Folic acid orally, 10 mg daily, was given for two months There was no change in the blood values, and the patient stated that numbness and tingling had become more severe, although there was no demonstrable alteration in the neurologic signs Folic acid was discontinued, and refined liver extract, 15 units, was given intramuscularly, at first twice and later once weekly The blood values were entirely normal, and there was a moderate degree of relief of neurogenic symptoms one month later

In the other 2 remaining cases, there can be no doubt that spinal cord disease progressed actively while the patients were receiving reasonably large doses of folic acid One of these (J N) a man of 51 years, was found to have histamine refractory achlorhydria and atrophic gastritis by gastroscopic examination four years before the diagnosis of pernicious anemia was made At the earlier examination his complaints were limited to gastrointestinal disturbances, he had no glossitis, no anemia, and no symptoms referable to the nervous system In April 1946 he was admitted to another hospital where the diagnosis of pernicious anemia was made Symptoms of increasing fatigability, paresthesia of the hands and feet, and ataxia had been present for about six months Shortly before his admission, he developed pronounced mental changes characterized by depression, feelings of guilt, and religious preoccupation Details of the hematologic and neurologic examinations at the time of admission are not available, but it is known that the anemia was of moderate degree with an erythrocyte count of approximately 3,000,000 per cu mm The patient was able to walk unassisted, and there were no sphincter disturbances He received one or two injections of liver extract, and then was treated exclusively with folic acid, 20 mg by mouth daily There was symptomatic improvement with clarification of the mental status, and he was discharged after about one month in the hospital, in May, 1946 He continued to take folic acid in the above dosage at home, and the fact that he actually received the medication is attested by his wife who is an entirely reliable person, well known to us On June 14, 1946, he was first seen in the out-patient department of the Simpson Memorial Institute He walked alone, but with considerable ataxia, was oriented and responsive, and had no specific complaints other than paresthesia His erythrocyte count was 3,400,000 per cu mm, hemoglobin 11.9 grams per 100 cc, and hematocrit 35 per cent He was advised to continue taking folic acid, 20 mg daily On the morning of June 20 he was unable to leave his bed, and during the next two days he rapidly developed the signs of extremely severe spastic ataxic paraplegia with loss of sphincter control On June 22 he was admitted to the Simpson Memorial Institute Knee and ankle jerks were not obtained Plantar stimulation gave an extensor response bilaterally Vibratory sense was completely lost over the bones of the lower extremities and the crests of the ilium Sense of motion and position of the toes was absent The patient was unable to bear any weight on his legs and had no sense of floor resistance Folic acid was discontinued, and refined liver extract, 15 units, was given daily by intramuscular injection This dosage was continued until August 5, when it was reduced to 15 units, three times a week, until December 12, when it was changed to 15 units twice weekly Improvement of the neurologic status was slow but definite After six weeks he was able to use a walking device, and sphincter control had returned In four months he could get about with crutches, and nine months after the institution of liver extract therapy he discarded the crutches for canes At that time, March, 1947, the knee and ankle jerks had returned, the Babinski sign was no longer obtained, and vibratory sense was present, although diminished as far distal as the mid-tibiae Hematologic values were restored to normal within a few weeks after beginning liver therapy

The last case to be reported is that of a man (S K) of 55 years who was admitted to our service on July 12, 1946 His earliest symptom was impaired sense of taste (? olfactory disturbance) and anorexia which developed eleven months before his admission One month later he first noted numbness and tingling of the extremities and difficulty in walking His symptoms were very slowly progressive, and he

continued with his occupation as a merchant until his hospital admission. The initial red blood cell count was 3,600,000 per cu mm, hemoglobin 12.2 grams per 100 cc, hematocrit 36 per cent. Histamine refractory achlorhydria was present. The gait was ataxic, Romberg and Babinski signs were present, knee and ankle jerks were hyperactive bilaterally, vibratory sense was impaired to absent over the lower extremities, and sense of motion and position of the toes was disturbed but not completely lost. A diagnosis of pernicious anemia with moderately advanced postero-lateral column degeneration was made, and the patient was treated with folic acid, 10 mg intramuscularly daily. After ten days the oral route was substituted for the intramuscular mode of administration. The patient was discharged on July 28 and walked out of the hospital unassisted. He returned eleven days later, on August 9, unable to stand or walk alone and with complete loss of vibration and position sense in the lower extremities. The erythrocyte count at this time was 3,900,000 per cu mm, hemoglobin 14.6 grams per 100 cc, hematocrit 39 per cent. In place of folic acid he was given refined liver extract, 15 units intramuscularly daily for one week, then 15 units three times a week. He returned four weeks later, on September 9, showing some improvement, but unable to walk without assistance. At his next visit, after another interval of four weeks, he walked alone with the aid of a cane. On June 17, 1947, after ten months of liver extract therapy, he felt quite well and walked with only a slightly hesitant gait. Vibratory sense was apparently normal over the right lower extremity, but was diminished, although nowhere absent, over the left. There was return of sense of motion and position of the toes.

### DISCUSSION

Experience in the management of cases of pernicious anemia during the early period of their treatment and over a number of years indicates that administration of sufficient amounts of desiccated stomach, whole liver, oral liver extract, and crude or refined liver extracts by parenteral routes, if accompanied by a hematologic response, will invariably lead to the arrest of the neurologic degenerative process and will usually be followed by a significant degree of symptomatic and functional improvement. Furthermore, if adequate treatment, as judged by consistently normal erythrocyte values, is taken continuously, exacerbation of nervous system disease will not occur. Even if treatment is irregular and suboptimal, provided there are no long periods of relapse, patients with less severe degrees of neurologic involvement will rarely suffer irreversible progression of their neural lesion. These statements are apparently not valid in the case of folic acid therapy. The occurrence and progression of combined system degeneration in patients with pernicious anemia under treatment with folic acid was first reported by Vilter, Vilter and Spies<sup>10</sup> and by Meyer.<sup>11</sup> The phenomenon has also been noted by Heinle and Welsh<sup>12</sup> and by Hall and Watkins.<sup>13</sup> In some of the cases reported, the activity of the process was not arrested when the dosage of folic acid was increased many times.

The failure of folic acid to control spinal cord disease in some cases may be taken as evidence that this vitamin corrects only a specific deficiency responsible for the hematopoietic disturbances occurring in pernicious anemia. This hypothesis, however, does not satisfactorily explain two types of observations which have been made by ourselves, as well as by others, namely, first, the fact that not all patients with pernicious anemia, in the absence of important complications, respond to folic acid by restoration of fully normal hematologic values, even when large doses of the vitamin are given by both oral and parenteral routes, and second, some patients experience definite relief of neurogenic symptoms while receiving folic acid. This improvement may be due primarily or entirely to peripheral nerve

recovery, but even so it may be said that such patients, over a period of many months, show no evidence of progress of nervous system involvement

The fairly uniform and predictable results of liver and stomach therapy in pernicious anemia and the variability of the responses of persons with this disease to administration of synthetic folic acid suggest that there is in pernicious anemia a widespread metabolic defect in which a number of interrelated factors or processes are involved, one of these being the ability to convert the naturally occurring conjugated form of folic acid to the free vitamin <sup>14</sup> <sup>15</sup> However, defective utilization of folic acid is not the only cause either of the hematopoietic or the neural disturbances. It appears, with some supportive evidence,<sup>14</sup> that among the therapeutic properties of stomach and liver is included a corrective effect on the disordered metabolism of folic acid which is responsible, in part, for the manifestations of pernicious anemia

### SUMMARY AND CONCLUSIONS

Seventy patients with pernicious anemia have been observed for periods of not less than ten years. The clinical course in these cases has been analyzed with particular reference to changes in the neurologic status

Most of the patients, whether treated with oral preparations of stomach or liver, or parenteral crude or refined liver extracts, showed significant improvement of their neurologic manifestations. The period of improvement was limited, essentially, to the first year of therapy

Thirty-six members of the series received treatment regularly and were maintained consistently in complete hematologic remission. Fifteen of the patients did not adhere to an optimal therapeutic regimen, and their blood values were frequently abnormal, although definite relapses did not occur. In the former group there were no instances of development or progression of neural lesions. In the latter such adverse changes as did occur were transient and reversible on resumption of adequate therapy. Nineteen patients in the series suffered clinical and hematologic relapses after their initial response to intensive therapy. The end results in this group were not so favorable, but nevertheless serious progression of spinal cord involvement was rarely observed. The apparent infrequent occurrence of pronounced changes is attributed to the short duration of the relapses and to the relatively mild degree of nervous system involvement present when the diagnosis of pernicious anemia was made. It may be assumed that patients with more extensive neural disease who suffered relapses, progressed to a fatal termination

The observations reported in no way justify the conclusion that irregular or sub-optimal therapy is without serious risk. They are presented in order to indicate what the long-term clinical results may be in the case of patients with pernicious anemia, who frequently fail to adhere to an ideal therapeutic regimen

The early results of treatment with synthetic folic acid, as observed in a series of 15 patients, indicate that both the hematologic and neurologic response to this form of therapy is much less predictable than is the case with stomach or liver preparations. It is suggested that disturbance of folic acid metabolism is not the sole cause of either the hematologic or the neurologic manifestations of pernicious

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anemia, but that inability to utilize folic acid effectively may play a part in the development of both myeloid and neural abnormalities

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# THE DEVELOPMENT AND PROGRESSION OF SUBACUTE COMBINED DEGENERATION OF THE SPINAL CORD IN PATIENTS WITH PERNICIOUS ANEMIA TREATED WITH SYNTHETIC PTEROYLGLUTAMIC (FOLIC) ACID

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THE ISOLATION, identification and synthesis of folic acid (pteroylglutamic acid) have provided an extremely potent hematopoietic agent.<sup>1</sup> Unquestionably folic acid induces hematologic remissions in patients with pernicious anemia. Evidence is accumulating that indicates its ineffectiveness in preventing the development or progression of subacute combined degeneration of the spinal cord. Furthermore, it is not certain that normal blood levels can be maintained for prolonged periods of time in patients treated with folic acid alone.

During the last seventeen months we have substituted synthetic folic acid† for liver extract in the treatment of 22 patients with pernicious anemia. These cases have been observed with extreme care for evidences of development or progression of neurologic complications and for changes in hematologic or clinical status. During this brief period of time significant changes have developed in many of these patients that make advisable a report of our observations at the present time.

## METHOD OF STUDY

### CLINICAL MATERIAL

The diagnosis in each case was established by the demonstration of a macrocytic hypochromic anemia with an associated leucopenia and thrombocytopenia, a histamine-refractory gastric achlorhydria and a response to liver extract or to folic acid with reticulocytosis and restoration of normal blood values. In many cases a megaloblastic bone marrow typical of pernicious anemia was demonstrated before treatment was started. Four patients were in hematologic relapse and were hospitalized during the initial period of study. Two of these patients previously had been seen in hematologic relapse, and remission had been induced with liver extract. It has been possible to compare their responses to folic acid with those previously obtained with liver extract. One patient in relapse had severe active subacute combined degeneration of the spinal cord.

The patients in remission had been known to have pernicious anemia for periods ranging from one to nineteen years and had been treated with liver extract in the Outpatient Department of the Massachusetts Memorial Hospitals before the beginning of this study. Six of these patients had evidence of subacute combined degeneration as manifested by paresthesias, diminution of vibration sense or reflex changes at the beginning of the study, but in all these cases the disease had been arrested and the signs had been unchanged for several years. Liver extract therapy was stopped at the beginning of this study. There was no restriction of diet, which was considered to be adequate in all patients. Eleven patients were taking supplementary vitamins or yeast, and these were allowed to continue this medication during the period of study. Folic acid was administered orally in 16 cases in doses ranging from 1.25 to 15.0 mg. daily. Six patients received 30 to 100 mg. by intramuscular injection once every four weeks.

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\* Formerly Welch Fellow in Internal Medicine of the National Research Council. A portion of this investigation was carried out while under tenure of the Welch Fellowship.

† The synthetic folic acid (pteroylglutamic acid) used in this study was kindly furnished by Dr. Stanton M. Hardy of the Lederle Laboratories, Inc., Pearl River, N. Y.

All the patients were seen at least monthly in the Outpatient Department of the Massachusetts Memorial Hospitals and when it became evident that neurologic symptoms were developing they were seen at weekly or semiweekly intervals. Careful neurologic and hematologic studies were performed at each visit.

### TECHNICAL METHODS

Blood studies were performed on venous blood placed in mixed ammonium and potassium oxalate.<sup>2</sup> Hemoglobin determinations were done on photoelectric colorimeters by the oxyhemoglobin method.<sup>3</sup> Hematocrits were determined with Wintrobe tubes, with centrifuging for one hour at a relative centrifugal force of 1800. Erythrocyte and leucocyte counts were made in duplicate and averaged. Erythrocyte indices were calculated by the method of Wintrobe. Direct platelet counts were made with Rees-Ecker diluting fluid. Reticulocyte counts were made on blood films prepared from a mixture of venous blood with 0.3 per cent cresyl blue and 0.6 per cent solution of sodium chloride. Films of bone marrow obtained by sternal aspiration were stained with Wright's and Giemsa's stain.

### OBSERVATIONS

#### PERNICIOUS ANEMIA IN RELAPSE

Four patients were in severe hematologic relapse when folic acid therapy was started. Of these, 2 had never before had antianemia therapy, 1 was in relapse subsequent to three years without liver extract therapy, and 1 was in relapse following a low maintenance dose of folic acid. The courses of these 4 patients are typical of the responses that may be produced with folic acid, and brief case histories are presented.

#### CASE I

*Pernicious anemia in severe hematologic relapse. Remission induced with orally administered folic acid. Gradual development of anemia and subacute combined degeneration of the spinal cord after one year of maintenance on folic acid.*

J B, a 65 year old white man, was admitted to the hospital in May, 1946, with a history of easy fatigability for two years and marked weakness and anorexia for two weeks. Physical examination revealed pallor of the skin and mucous membranes, a red tongue with atrophy of the lateral papillae, hypoactive deep tendon reflexes in the lower extremities and slightly diminished vibration sense in the feet. Blood studies showed a severe macrocytic anemia, leucopenia and thrombocytopenia (table 1 and fig 1), and the sternal bone marrow was characteristic of pernicious anemia in relapse. There was histamine refractory gastric achlorhydria. Folic acid therapy, 15 mg daily by mouth, was started at this time. Weakness and anorexia were noticeably lessened on the second day of therapy and the patient was completely asymptomatic at the end of the second week. In one month's time the physical findings were normal and there was full recovery of vibration sensation. A maximum reticulocyte response of 29 per cent was reached on the eighth day of therapy. Just prior to the rise in reticulocytes a substantial increase in the white cell and platelet counts was noted. A steady increase in the red cell count and hemoglobin began after the fourth day. Normal blood levels and red cell indices were present by the third month and were maintained until the eighth month, when the red cell count, hematocrit and hemoglobin began to fall. By the twelfth month the patient showed a definite anemia.

With the exception of occasional slight glossitis the patient remained asymptomatic during the first eleven months of folic acid therapy. Beginning with the seventh month there was gradually progressing diminution in vibration sense, although position sense and motion sense were normal and Romberg's sign was negative. In the twelfth month the patient began to experience stiffness of the feet and numbness of the fingers. His gait became unsteady, particularly in the evening, and he staggered when he attempted to walk in the dark. Knee and ankle jerks were markedly hypoactive at this time, vibration sense was

impaired below the knees, and motion sense was diminished in the toes. The patient swayed slightly in the Romberg position and staggered when he tried to walk with his eyes closed.

*Comment* This previously untreated case of pernicious anemia showed early and rapid clinical and hematologic improvement in response to 15 mg of folic acid given daily by mouth. The reticulocytosis of 29 per cent in response to folic acid therapy was slightly less than the 37 per cent expected optimum response to liver extract, but the rate of regeneration of erythrocytes, leucocytes and platelets was fully as rapid as would have been expected with liver extract therapy. The blood remained normal for eight months but subsequently fell to anemic levels. Definite subjective and objective signs of subacute combined degeneration appeared during the twelfth month of folic acid therapy.

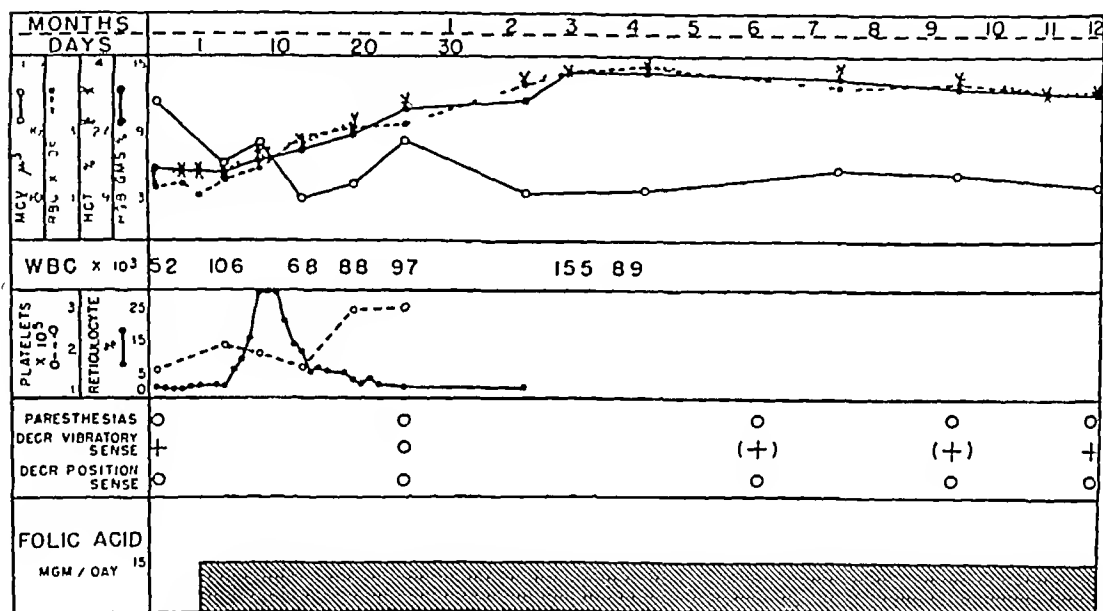


FIG 1 (Case 1) Previously untreated pernicious anemia in relapse. Remission induced with orally administered folic acid. Gradually developing anemia and subacute combined degeneration after 7 months of folic acid therapy.

## CASE 2

*Pernicious anemia with mild anemia but severe subacute combined degeneration. No improvement in neurologic status after twenty-five days of orally administered folic acid.*

A W, a 60 year old white woman, was admitted to the hospital with a history of increasing paresthesias in all extremities, weakness, anorexia and weight loss of one year's duration. The significant physical findings were slight atrophy of the lateral lingual papillae, hyperactive deep tendon reflexes, a positive Romberg's sign, a slightly ataxic, spastic wide-based gait, marked diminution of vibration sense in the knees, ankles, toes and fingers and some impairment of position sense in the fingers. The red cell count was 3,000,000, the hemoglobin 12.8 Gm per 100 cc, the mean corpuscular volume 122 cubic micra, and the white cell count 5700. There was histamine-refractory gastric achlorhydria. Folic acid was administered orally in a daily dose of 15 mg throughout the twenty days of hospitalization. The reticulocytes rose from 2.1 to 5.8 per cent on the seventh day. At discharge the red cell count was 3,410,000, the hemoglobin 13.9 Gm per 100 cc, and the white cell count 8275. After one week during which the

folic acid dosage was increased to 45 mg daily the red cell count was 3,540,000 and the hemoglobin 14 Gm per 100 cc

After twenty-eight days of folic acid therapy paresthesias and weakness persisted unchanged, and there was no improvement in the signs of subacute combined degeneration of the spinal cord. Daily intramuscular injections of 15 units of purified liver extract were begun and continued during the next five weeks. Subjective improvement and a decrease in paresthesias occurred in response to this therapy, but further neurologic examinations could not be performed. A blood study performed after two months of folic acid therapy and thirty-eight days of liver extract therapy showed a normal hemoglobin and hematocrit but persistent marked macrocytosis (table 1)

*Comment* This previously untreated patient had a moderate, although markedly macrocytic, anemia but severe subacute combined degeneration of the spinal cord. She had a submaximal reticulocyte response to folic acid (only 5.8 per cent as compared with an anticipated 12.0 per cent), and the blood levels rose very slowly even after liver extract therapy was given in addition to an increased dosage of folic acid. There was no subjective or objective evidence of improvement of the subacute combined degeneration during twenty-eight days of folic acid treatment, and it was not possible to follow the patient adequately for a longer period of time.

### CASE 3

*Pernicious anemia in relapse, remission induced with orally administered folic acid. Subacute combined degeneration and mild anemia developing after sixteen months of maintenance therapy with folic acid. Progression of subacute combined degeneration after institution of liver extract therapy while folic acid was continued.*

H C., a 59 year old white woman, was first admitted to the hospital in December, 1940, with weakness, glossitis, anorexia and weight loss of two years duration. Physical examination revealed marked pallor of the skin and mucous membranes, atrophy of the lingual papillae, moderate glossitis, a palpable liver and diminished vibration sense below the knees. There was a severe macrocytic anemia and a megaloblastic bone marrow (table 1 and fig. 2). Histamine-refractory gastric achlorhydria was present. In response to 15 units of liver extract administered intramuscularly daily, there was a rise in reticulocytes to 14.6 per cent. After one month of therapy the red cell count was 4,330,000 and red cell indices were essentially normal. Therapy was gradually reduced to 15 units of liver extract a month. Blood values continued to rise and throughout the ensuing seventeen months were optimal.

Except for the occasional ingestion of cooked liver the patient lapsed in therapy from September, 1942, to January, 1946, when she was readmitted to the hospital with recurrence of the original symptoms and paresthesias of the hands and feet. The significant physical findings were pallor of the skin and mucous membranes, atrophy of the lateral lingual papillae, a slightly unsteady gait, hyperactive knee jerks, absent ankle jerks and a moderate decrease in vibration sense below the knees. The blood and bone marrow were characteristic of pernicious anemia in relapse (table 1 and fig. 2). The patient was given 15 mg of folic acid by mouth daily, and a maximum reticulocyte response of 11.8 per cent was obtained on the sixth day of therapy. She rapidly became asymptomatic, and after one month of treatment the red cell count and hemoglobin had risen to moderate levels with a decrease in macrocytosis. There was no change in neurologic status at that time. Folic acid therapy (15 mg by mouth daily) was continued after discharge in February, 1946. One month later the patient was readmitted for treatment of a myocardial infarction and pyelitis. Vibration sense in the ankles and toes was diminished. The blood values were unchanged. The dosage of folic acid was increased to 100 mg daily, by mouth for five days and then intravenously for the next ten days. This increased dose produced no improvement in the blood picture, and the original dosage of 15 mg daily by mouth was resumed. Nineteen days later this was supplemented by daily intramuscular injections of 15 units of liver extract for twelve days. This resulted in symptomatic improvement, relief of the glossitis and improvement in vibration sense in the ankles and toes, but no



further hematologic response. During the subsequent two months on folic acid therapy alone the red cell count and hemoglobin rose to fairly satisfactory levels and the red cell indices became normal. Vibration sense was fully recovered. During the ensuing ten months there was a gradual decrease of the red cell and hemoglobin levels and a return of macrocytosis.

In the sixteenth month of folic acid treatment and twelve months after the last injection of liver extract, the patient complained of steadily increasing numbness of the hands, forearms and feet. Her family stated that she became extremely forgetful and irritable. There was a rapid and progressive loss of vibration sense in the lower extremities and impairment of position and motion sense in the toes and fingers. Liver extract therapy was started, 75 units being given first semiweekly and then weekly, and folic acid medication was continued. In spite of the injection of 260 units of liver extract during the next six weeks the symptoms and signs of subacute combined degeneration of the spinal cord progressed. At the end of this period folic acid therapy was discontinued.

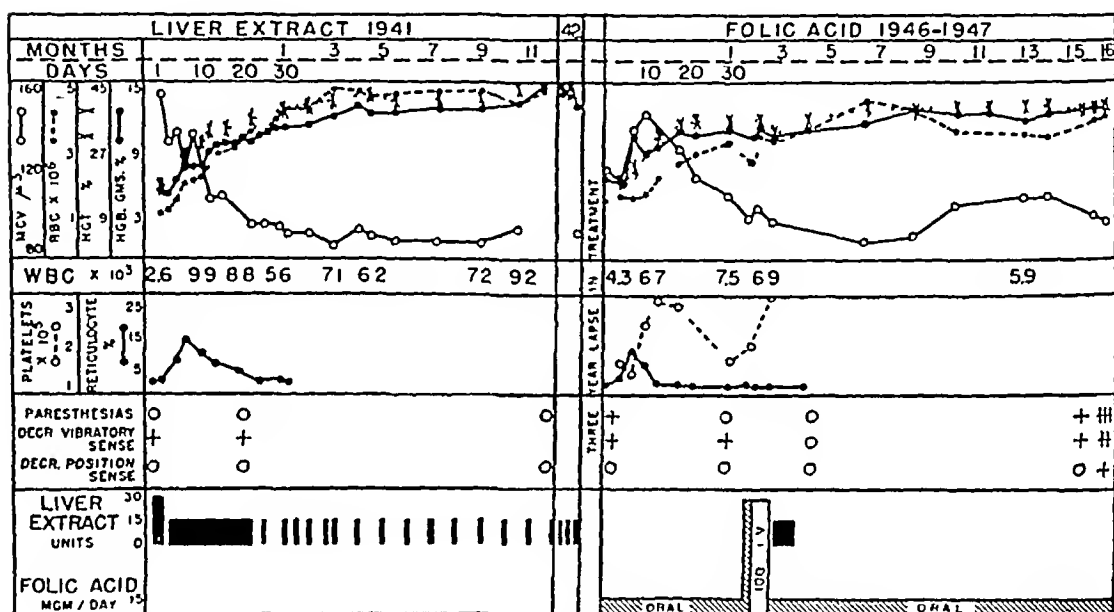


FIG. 2 (Case 3) Pernicious anemia with comparable remissions induced on different occasions with liver extract and with folic acid. Suboptimal response to oral folic acid not improved by large doses of folic acid administered intravenously or by liver extract. Progression of subacute combined degeneration 12 months after last injection of liver extract.

*Comment* This case provides an interesting comparison of the relative effectiveness of folic acid and liver extract in inducing remission in pernicious anemia. Symptomatic improvement occurred earlier and was more marked with liver extract than with folic acid therapy. The reticulocyte response to both liver extract and folic acid was submaximal. The rate of increase in the red cell count in response to folic acid was slower than had occurred previously with liver extract, but the hematologic response probably was hampered by the complicating myocardial infarction and pylephlebitis. In the face of these complications neither an increase in the dosage of folic acid nor a course of liver extract therapy was effective in improving the blood picture. Following recovery from these illnesses the blood levels temporarily rose to normal. The optimal levels previously maintained with liver extract therapy never were observed during folic acid treatment, and a gradual diminution of blood levels occurred during the last ten months of therapy. Subacute combined de-

generation became apparent in the sixteenth month of folic acid therapy, twelve months after the last injection of liver extract, and neurologic disease progressed during the next six weeks in spite of the injection of 260 units of liver extract

## CASE 22

*Hematologic relapse on 30 mg of folic acid administered once monthly by injection Remission on 15 mg of folic acid daily by mouth Subsequent explosive development of severe subacute combined degeneration of the spinal cord*

J S, a 69 year old white man, had been treated for pernicious anemia with liver extract by a private physician in 1938, and was first admitted to the hospital in 1939 with a history of weakness and anorexia following a six months lapse in therapy. Blood studies showed a severe macrocytic anemia (table 1 and

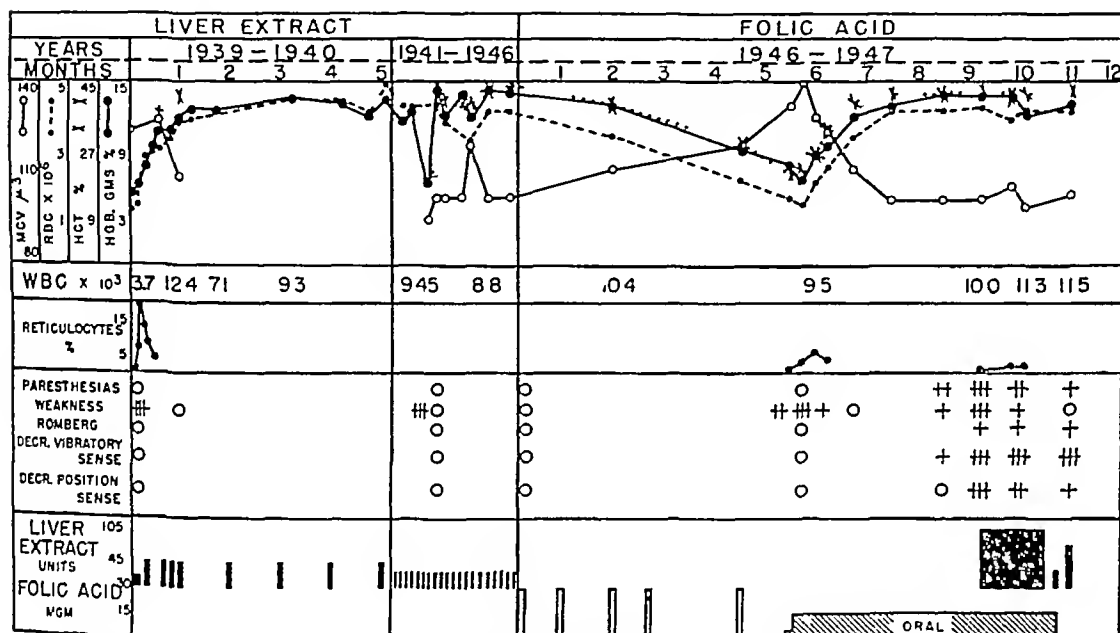


Fig 3 (Case 22) Relapse of pernicious anemia while receiving 30 units of folic acid by intramuscular injection once each month. Remission induced with 15 mg of folic acid daily by mouth comparable with remission induced with liver extract on previous occasions. Rapid development and progression of subacute combined degeneration on folic acid maintenance therapy while blood was normal.

fig 3) The patient responded to liver extract therapy with a reticulocyte response of 20.9 per cent on the sixth day of therapy, and normal blood levels were attained by the third month. In 1942 the patient again lapsed in therapy and underwent a second hematologic relapse. He again showed an adequate response to liver extract. Since 1943 adequate blood levels were maintained with intramuscular injection of 30 units of liver extract monthly. The patient ingested daily 10-15 Gm of yeast during the entire period of the subsequent study, a fact that should exclude vitamin deficiency as a contributing factor. Folic acid was substituted for liver extract in June, 1946, and 30 mg of folic acid once each month was given by intramuscular injection. At this time the patient was completely asymptomatic, physical examination was essentially negative, and neurologic examination was entirely negative. The blood levels were suboptimal, with a moderate degree of macrocytosis (table 1 and fig 3). There was a persistent drop in the red cell and hemoglobin levels, and by the fifth month the patient showed a marked macrocytic anemia. Weakness and dyspnea appeared during the third month, but the only abnormal physical finding was marked pallor of the skin and mucous membranes. Neurologic examination was completely negative. At this time therapy was changed to a daily oral dose of 5 mg of folic acid. Symptoms of anemia increased,

and there was a further decrease in blood levels during the next week. The dosage of folic acid was increased to 15 mg a day by mouth. Rapid symptomatic and hematologic improvement followed, and in two months the red cell count returned to its original pre-folic acid level. A reticulocyte response of 63 per cent was noted on the seventh day, but since these counts were performed only at weekly intervals the maximum reticulocytosis probably was not detected. Neurologic examination at this time was negative.

Eight and one-half months after folic acid had been substituted for liver extract, three months after the start of oral folic acid therapy and one month after restoration of normal blood levels, the patient complained of heaviness in the soles of his feet and stated that he felt muscle-bound in the calves and thighs. The only neurologic abnormality at this time was slightly diminished vibration sensation in the toes. One week later his hands and feet felt 'like blocks of wood' and there were periodic episodes of a burning sensation from the toes to the hips. At this time the patient began to need assistance in maintaining his balance, and soon he was unable to walk without considerable aid. Within another two weeks he was unable to stand or walk and could not feed himself because of incoordination of the hands.

On admission to the hospital the general physical examination, complete blood studies and sternal bone marrow examination yielded normal findings. The patient's mental status and speech were normal. Examination of the cranial nerves showed impairment in the first (inability to recognize the smell of coffee, vanilla or wintergreen) and the second (moderate bilateral diminution in visual acuity and moderate constriction of visual fields), although the optic fundi were not remarkable. The remaining cranial nerves were intact. There was no gross inequality in muscular volume or power, but there was a definite increase in muscle tone, spastic in type, of the arms and legs. Coordination was extremely poor, and the patient was unable to hold objects or to feed himself. There was astereognosis, even for large objects, and marked past pointing in both arms. The heel-to shin test was poorly done. Romberg's sign was positive, and the gait, even with assistance, was spastic, slapping and staggering. Deep tendon reflexes in the lower extremities were markedly hyperactive, more so on the left than on the right. The Babinski response was present on the left and there was no response to plantar stimulation on the right. Abdominal and cremasteric reflexes were absent. Vibration sense was entirely absent below the tenth thoracic segment and in both hands, wrists and elbows. Position and motion sense were impaired generally, most markedly in the fingers, toes and ankles. There was loss of light-touch sensation over the ulnar side of both hands and on the medial aspect of the right foot. There was irregular patchy diminution of pain and temperature sensation in both arms and both legs, and decrease in sweating of both lower extremities and complete absence of sweating from the level of the knees downward. There was no interference with bladder or bowel function, however, and no change in sexual function (the patient had been impotent for 10 years). A lumbar puncture yielded normal spinal fluid.

Injection of 105 units of concentrated liver extract (15 units per cubic centimeter) was given intramuscularly daily for the next five weeks, and the administration of 15 mg of folic acid daily by mouth was continued. During this period there was gradual subjective improvement, beginning on the eleventh day. The patient gradually regained the coordinated use of his hands and feet, and after three weeks of liver extract therapy he was able to feed himself and to walk unassisted and without staggering, although the gait was still spastic and slapping. He was discharged from the hospital at the end of five weeks, at which time he felt well except for some residual numbness of the hands and feet and stiffness of the knees. Coordination of movement was normal, but the gait showed slight slapping. During the period of hospitalization the pattern of neurologic improvement was striking. First there was a decrease in the signs of lateral column disease: muscle tone returned to normal, deep tendon reflexes became less hyperactive, the plantar responses became flexor, and there was a gain in strength. With this improvement the patient was able to walk and to feed himself, even though the posterior column signs still persisted. Position sense was slightly improved in the fifth week of liver extract therapy, but there was no return of vibration sense.

Following discharge the patient was given 75 units of liver extract intramuscularly once a week and the dosage of 15 mg of folic acid daily was continued. There was only slight improvement in position sense during the next three weeks, no improvement in vibration sense, and persistence of paresthesias. Folic acid was discontinued after two months of liver extract therapy. Two weeks later position sense was practically normal, vibration sense perception began to return, and the hands and feet felt much less numb and nearly normal.

*Comment* This patient gradually developed a hematologic relapse over a six month period during which treatment consisted of 30 mg of folic acid by injection once a month. The rate of development and the severity of the hematologic relapse were comparable to two previous relapses that occurred following omission of liver extract. This indicates that folic acid in the amount given had no effect in maintaining remission. The hematologic response to 15 mg of folic acid taken orally each day was excellent and was comparable with the response obtained on two previous occasions with liver extract.

The most striking features of this case were the explosive development of extremely severe subacute combined degeneration after nine months of folic acid therapy and its appearance at a time when the peripheral blood picture and bone marrow were normal. Improvement of symptoms and signs of lateral column disease occurred gradually following the addition of liver extract therapy, but vibration sense and numbness of the hands and feet improved only after discontinuation of folic acid.

The clinical and hematologic responses of 3 cases to folic acid therapy were excellent and quite comparable to those that would have been expected with optimum liver extract therapy. Indeed, in Case 22 the rate of blood regeneration under folic acid therapy almost exactly paralleled that previously obtained with liver extract (fig 3). The reticulocyte responses were slightly less than would have been expected with liver extract, but were quite adequate in all 3 cases. Increases in white cell and platelet counts were marked and rapid. In Case 2 the degree of anemia was not severe, and consequently the rate of blood regeneration was not rapid. The suboptimal blood levels eventually obtained in Case 3 were not improved by additional liver extract therapy and probably are a reflection of the complicating cardiac disease and pyelonephritis.

Cases 1, 3 and 22 showed improvement in the sense of well-being on the third or fourth day following the beginning of folic acid therapy. This improvement was neither so striking nor so marked as that usually seen following the exhibition of liver extract. Glossitis was present in Cases 1, 3 and 22 and improved very slowly under folic acid treatment. In Case 1 it occasionally recurred during the period of maintenance therapy.

The extremely marked signs of subacute combined degeneration in Case 2 showed no improvement during the twenty-eight days of folic acid therapy, but they did not progress. The numbness and slight diminution of vibration sense initially present in Cases 1 and 3 gradually improved over a period of several months with folic acid therapy but recurred again after a year's treatment. Case 22 showed no neurologic abnormality at the time of hematologic relapse, and it was not until three months later, at a time when the blood picture was normal, that the patient developed extremely severe and rapidly progressive subacute combined degeneration of the spinal cord.

#### MAINTENANCE THERAPY OF PERNICIOUS ANEMIA IN REMISSION

Twenty-one patients with pernicious anemia in remission have been maintained on folic acid therapy for periods ranging from eight to seventeen months. In 19





TABLE 1—Continued

[illegible]

17	E B	72	F	P A	9	30	Before 2 mo 6 mo 7 mo 10 mo 11 mo	100 100 1 25 15 15	monthly monthly daily daily daily	I M I M oral oral oral	3 59 3 68 3 11 3 39 3 14 3 40	12 1 12 2 11 9 11 9 11 7 12 6	38 0 37 5 36 0 35 5 34 5 37 5	106 102 116 105 110 110	6 5	N N N N N + N N N N N + N + + + + + N N N N N N N N N N N + N N N N N N
18	C W	46	F	P A	9	30	Before 3 mo 7 mo 9 mo 12 mo	100 1 25	monthly daily	I M oral	3 77 4 69 3 82 3 91	13 5 13 3 12 9 12 6	40 0 40 5 39 0 38 5	106 86 102 99	6 9	N N
19	J L	50	M	P A	5	30	Before 3 mo 7 mo 10 mo	100 100 100	monthly monthly monthly	I M I M I M	4 31 4 91 4 40 4 27	13 8 14 3 14 0 13 0	40 5 42 0 40 0 39 0	94 86 91 91		N N
20	M G	61	F	P A	8	30	Before 4 mo 6 mo 9 mo 12 mo	40 40 1 25 1 25	monthly monthly daily daily	I M I M oral oral	4 08 4 07 4 38 4 33 4 05	13 6 14 5 13 7 14 1 14 4	39 5 42 0 40 0 42 0 41 5	97 103 91 97 103	6 1	N N
21	C M	71	F	P A	4	30	Before 4 mo 5 mo 6 mo 12 mo	30 1 25 1 25 1 25	monthly daily daily daily	I M oral oral oral	4 46 4 21 4 25 4 77	14 2 14 5 14 6 15 0	44 5 44 0 46 0 46 5	100 105 108 97	9 2	N N
22	J S	69	M	P A	8	30	Before 5 1/2 mo 6 mo 8 mo 8 1/2 mo 9 1/2 mo	30 15 15 15 15 +105 units liver	monthly daily daily daily daily extract daily	I M oral oral oral oral I M	4 31 1 61 2 23 4 35 4 37 4 39	14 9 7 8 9 1 13 5 14 5 14 3	46 0 23 0 29 0 44 0 44 0 45 0	107 143 130 101 101 103	9 2 9 5 9 4 10 0	N N N N N N + N N N N N N + N N N N N N + N N N N N N + N N N N N N + N N N N N N + N N N N N N + N N N N N N +
							10 1/2 mo	15 +75 units liver	daily	oral	4 36	14 7	45 0	103	11 5	N N N N N N
							11 1/2 mo	folc acid omitted, 30 units liver	extract weekly	I M	5 31	15 6	48 5	91	10 5	N N N N N N
							11 1/2 mo	extract weekly 30 units liver	extract weekly	I M	5 47	16 9	51 5	94		N N N N N N



TABLE 1, ADDITIONAL REMARKS

- Case 1* Rapid remission induced. Maximum reticulocytosis of 29% on 8th day. Blood declined to sub-normal levels after 6th month. Probable development of mild S C D beginning in 7th month.
- Case 2* Mild anemia but marked macrocytosis. Maximum reticulocytosis of 5.8% on 7th day. Slight improvement in blood during 28 days of folic acid therapy. Normal hemoglobin level but persistent macrocytosis following liver extract therapy. No improvement in S C D with folic acid therapy.
- Case 3* Response to folic acid comparable with response to liver extract 5 years previously. Maximum reticulocytosis 11.8% on 6th day. Suboptimal blood levels, not improved with liver extract, probably because of complicating disease. Evidence of S C D at 16 months. Rapid progression of S C D in spite of institution of liver extract therapy during continuation of folic acid.
- Case 4* Blood levels higher and macrocytosis less than when under liver extract therapy. S C D beginning in 11th month and progressing rapidly in spite of liver extract therapy while folic acid continued.
- Case 5* For 8 months blood levels much higher than with liver extract therapy. Moderate decrease after 8th month. No definite evidence of S C D.
- Case 6* Blood levels decreased at 8 months. No evidence of S C D.
- Case 7* Blood levels improved initially. Subsequently decreased and fluctuated in fashion similar to that observed during liver extract therapy. No evidence of S C D.
- Case 8* Blood levels same as with liver extract therapy. Probable development of early S C D.
- Case 9* For 8 months blood levels much higher than with liver extract. Then gradual decrease to initial levels. No evidence of S C D.
- Case 10* Blood levels initially higher than with liver extract, subsequent decrease. Definite development of S C D.
- Case 11* Blood levels same as with liver extract. No change in S C D.
- Case 12* Blood levels better than those maintained with liver extract. Progression of S C D.
- Case 13* Blood improved initially, but decreased to previous levels in 10th month. Definite progression of S C D in 12th month.
- Case 14* Blood levels unchanged even after increase in folic acid dosage. S C D developed at 9½ months. Progressed rapidly when folic acid dosage increased to 15 mg. Continued progression of S C D after institution of liver extract while folic acid continued.
- Case 15* Blood unchanged. Signs of S C D appeared in 7th month and progressed in spite of increased folic acid dose.
- Case 16* Blood unchanged. No evidence of S C D.
- Case 17* Decrease in blood levels during period of monthly injections. Slight improvement on daily oral dose of folic acid. Evidence of S C D appeared in 2nd month and progressed in spite of increased dose of folic acid.
- Case 18* Temporary decrease in macrocytosis. No evidence of S C D in spite of very low dose of folic acid.
- Case 19* Transient increase in blood levels. No evidence of S C D. Died of heart failure in 11th month.
- Case 20* Blood levels comparable with those maintained with liver extract. Patient required 11 months to relapse after omission of liver extract on a previous occasion. No S C D.
- Case 21* Blood levels comparable with those maintained with liver extract therapy. Improved with oral folic acid therapy. No S C D.
- Case 22* Hematologic relapse during monthly injections. Remission induced with oral folic acid. Rapid onset and progression of S C D when blood was normal. Very gradual and incomplete improvement with liver extract therapy while folic acid continued. More rapid improvement after omission of folic acid.

patients (including Case 22, previously described) the clinical and hematologic aspects of the disease had been well controlled with liver extract for from one to nineteen years prior to the substitution of folic acid therapy. In 2 patients (Cases 1 and 3) folic acid-induced remissions preceded maintenance therapy. This group has

been subdivided according to the maintenance dose of folic acid administered (table 2) An increase in dosage was made in 8 patients during the latter half of the period of observation, and specific reference to these changes will be made The hemato-

TABLE 2 —Results of Maintenance Therapy with Synthetic Folic Acid

Folic Acid Dosage	No of Patients	Duration of Therapy	Hematologic Status				Neurologic Status								
			No change	Persistently higher blood levels than with liver extract	Initial higher blood levels decreasing after 6 months	Re-lapse	No change		Progression or Development of Subacute Combined Degeneration						
									Probable			Definite			
							No	Patients	No	Patients	Time of onset	No	Patients	Time of onset	
mg		mo													
Oral (daily)															
15 0	5	8-17		# 4	# 1, # 3, # 5, # 6		2	# 5 # 6				3	# 1 # 3 # 4	11 16 12	
10 0	2	12	# 8*		# 7		1	# 7	1	# 8	11				
5 0	2	12			# 9 # 10		1	# 9				1	# 10	12	
2 5	1	11	# 11				1	# 11							
1 25	5	9½-11	# 16	# 12			1	# 16	1	# 12	12				
(Increased to 15)	(3)	(1½-2½)	# 14 # 15		# 13				2	# 13 # 15	12 7	1	# 14	9½	
Intramuscular injection (monthly)															
100	3	7-10			# 19		1	# 19							
(Changed to oral daily)															
1 25 at 9 months	(1)	3			# 18		1	# 18							
1 25 at 7 months	(1)	5	# 17									1	# 17	6	
15 0 at 10 months	(1)														
40	1	9													
(Changed to oral daily)															
1 25 at 9 months	(1)	3	# 20				1	# 20							
30	2	5-6				# 22									
(Changed to oral daily)															
1 25 at 5 months	(1)	7	# 21				1	# 21							
15 0 at 6 months	(1)	6										1	# 22	8½	
Totals	21		8	2	10	1	10		4			7			

\*Figures preceded by # are numbers of cases listed in Table 1

logic and clinical data before and during folic acid therapy are presented in tables 1 and 2

*Oral maintenance dose of 15 mg daily* Five patients (Cases 1, 3, 4, 5 and 6) were given a daily oral maintenance dose of 15 mg of folic acid With the exception of 1 patient (Case 6) who was treated for only eight months, these patients remained on the same dosage schedule for twelve months Satisfactory blood levels were ob-

served early in the course of treatment in all 5 patients, and 1 patient (Case 4) consistently maintained higher levels with folic acid than he did with liver extract. A decline in the red cell count and hemoglobin to suboptimal levels began after the fourth and sixth month, respectively, in 2 patients (Cases 1 and 3) in whom remission had been induced with folic acid. A similar drop in blood levels was not observed in the other 2 patients (Cases 5 and 6) until the eighth month. In 1 patient (Case 3) a significant macrocytosis developed concomitantly with the decline in blood levels.

All 5 patients felt extremely well and were asymptomatic during the first eight to eleven months of treatment. In the twelfth month of therapy definite signs of neurologic disease appeared in Case 1 (see case report). In Case 3, in the sixteenth month of folic acid therapy and twelve months after the completion of a supplementary course of liver extract therapy, the patient developed the symptoms and signs of neurologic disease (see case report).

In Case 4 there had been mild but quiescent subacute combined degeneration for at least eight years, and there was no change in this condition during the first ten months of folic acid therapy. Beginning in the eleventh month, however, the patient developed paresthesias of the hands and feet and weakness and stiffness of the legs. His family stated that he became very irritable and occasionally wept because of anger. By the twelfth month these symptoms were worse and there was unsteadiness of gait and difficulty in walking. Knee jerks were increased, and vibration sense was decreased at and below the iliac crests. Liver extract was started in addition to continuation of folic acid, but in spite of the injection of 260 units in the next three weeks there was rapid progression of neural disease. By the end of this time the patient was unable to walk because of weakness of the legs and unsteadiness of gait. He complained of warmth of the hands and feet. He showed increased muscle tone in the legs, markedly hyperactive knee jerks, absent left ankle jerk and decreased right ankle jerk, a positive Babinski sign on the left and normal plantar response on the right. Position and motion sense of the toes was poor. The heel-to-knee test was poorly performed. Vibration sense was absent below the iliac crest. Romberg's sign was markedly positive. Folic acid was discontinued and the patient was hospitalized. A lumbar puncture yielded normal fluid.

This patient developed progression of subacute combined degeneration during folic acid therapy, which proceeded rapidly in spite of parenteral injection of large amounts of liver.

*Oral maintenance dose of 10 mg daily.* Two patients (Cases 7 and 8) were given 10 mg of folic acid daily for a period of twelve months. Case 8 maintained constant blood levels. Case 7 showed an initial improvement in red cell count and hemoglobin but a persistent macrocytosis, and the blood subsequently returned to levels essentially similar to those observed during liver extract treatment.

In Case 8 progressive diminution of vibration sense in the lower extremities and fingers appeared during the eleventh month, and in the twelfth month Romberg's sign became positive.

*Oral maintenance dose of 5 mg daily.* Two patients (Cases 9 and 10) were maintained on 5 mg of oral folic acid daily for twelve months. In both patients there was an

initial increase in the red cells and hemoglobin and a decrease in macrocytosis, but in the eleventh and twelfth months the red cell count and hemoglobin returned to pre-folic acid values, and in Case 9 there was recurrence of macrocytosis

Subjective improvement and an increase in appetite were noted by each patient with beginning of folic acid therapy, and these continued throughout the period of observation. Case 9 remained asymptomatic and maintained a normal neurologic system during the period of observation. Paresthesias and diminution in vibration sense appeared in Case 10 in the eleventh month. By the twelfth month there was marked diminution of vibration sense in the toes and ankles, a positive Romberg's sign, a slightly unsteady gait, hypoactive knee and ankle jerks and a positive Babinski's sign.

*Oral maintenance dose of 2.5 mg daily.* Only one patient (Case 11) was given a daily oral dose of 2.5 mg of folic acid. This patient maintained as high blood levels throughout the eleven months of treatment as she had previously done with large doses of liver extract. She remained asymptomatic and showed no change in her neurologic signs of minimal subacute combined degeneration of the spinal cord.

*Oral maintenance dose of 1.25 mg daily.* Five patients (Cases 12-16) were given daily doses of 1.25 mg of folic acid by mouth. This dosage was increased to 15 mg daily in 3 patients (Cases 13, 14 and 15) in approximately the tenth month of treatment. With the exception of Case 16, who was observed for only eight months, the members of this group remained on folic acid for eleven, twelve or thirteen months. The blood picture of 3 of these patients was comparable to that previously maintained by them on liver extract. In Case 12 the blood levels were better, and in Case 13 there was a temporary increase in red cells and hemoglobin and a decrease in macrocytosis, which persisted for eight months and then returned to the levels previously maintained with liver extract.

Two of these patients (Cases 12 and 13) had subacute combined degeneration of the spinal cord at the time folic acid therapy was started, but in both the disease had remained stationary for several years under liver extract therapy. In Case 12 progression of neural disease was shown during the twelfth month of folic acid therapy. Weakness and stiffness of the legs, numbness of the feet, unsteadiness in gait and swaying on Romberg's test became definitely severer at this time and led to institution of liver extract therapy. In Case 13 the patient complained of a squeezing sensation in the feet and calves, and there was a definite decrease in vibration sense below the level of the iliac crests and a markedly positive Romberg's sign during the twelfth month of therapy.

After nine and one-half months of folic acid treatment the patient in Case 14 showed development of subacute combined degeneration. The dosage of folic acid was increased to 15 mg daily by mouth for the next six weeks, but the signs of neurologic disease progressed even more rapidly. By the eleventh month of maintenance therapy the patient exhibited weakness of the entire body and a numbness and tingling of the hands and feet. Knee and ankle jerks were extremely hyperactive, vibration sense was markedly impaired below the iliac crests and was entirely absent in the left leg. An injection of 75 units of liver extract was given, which produced only minimal improvement in one week's time. Folic acid was

omitted and 45 units of liver extract were given each week for the ensuing two weeks. Two weeks after discontinuance of folic acid there was marked subjective improvement, Romberg's sign was negative, and vibration sense was improved. There was strikingly rapid progression of neural disease during folic acid treatment, and significant improvement did not occur in spite of liver extract therapy until folic acid was discontinued.

Of the 2 other patients without subacute combined degeneration at the beginning of folic acid therapy, Case 16 remained asymptomatic and developed no abnormal neurologic signs during the eight months that she was observed. Case 15, however, showed progressive diminution of vibration sense from the seventh to twelfth month. An increase in folic acid to 15 mg daily at nine and one-half months failed to prevent progressive diminution of vibration sense or the development of hypoaffective knee jerks.

*Maintenance therapy with monthly intramuscular injections.* The remaining 6 patients (Cases 17-22) were given intramuscular injections of folic acid once a month. None of these patients had evidence of subacute combined degeneration at the time folic acid therapy was started. The doses administered were 100 mg (Cases 17, 18 and 19), 40 mg (Case 20) and 30 mg (Cases 21 and 22). With one exception (Case 19), oral folic acid therapy (1.25 to 15.0 mg daily) was substituted for injected folic acid in these patients between the fifth and ninth month of observation. The members of this group remained on folic acid for periods of ten to twelve months.

Of the 3 patients receiving 100 mg of folic acid monthly, 2 (Cases 18 and 19) showed an initial rise in blood levels with a subsequent decrease to the original levels. The blood levels of the other patient (Case 17) dropped slightly, but returned to the original suboptimal levels after substitution of a daily oral dose of 15 mg of folic acid.

This latter patient began to show paresthesias and diminution of vibration sense after the second month of therapy. The paresthesias decreased slightly following the administration, from the fifth month onward, of 15 Gm of Brewer's yeast daily. Daily oral doses of 1.25 mg of folic acid were substituted for the monthly intramuscular injections in the sixth month, and this dosage was increased to 15 mg daily in the tenth month. All these measures failed to halt progressive diminution of vibration sense. By the eleventh month there was marked diminution of vibration sense in both lower extremities. Motion and position sense were impaired in the toes, and Romberg's sign was positive. Case 18 remained asymptomatic and was placed on a daily oral dose of 1.25 mg of folic acid in the ninth month. In Case 19 there was no evidence of neurologic disease, but the patient died of congestive heart failure during the eleventh month.

One of the 3 patients on maintenance therapy of 30-40 mg monthly (Case 22) experienced a hematologic relapse within six months (see case report). A satisfactory hematologic remission was produced when orally administered folic acid was substituted for the monthly injections. The other 2 patients showed persistent macrocytosis, although their blood levels were comparable with those previously maintained with liver extract therapy. A moderate improvement in blood levels and a decrease in macrocytosis was observed in Case 21 after the substitution of a

daily oral dose of 1.25 mg of folic acid, but no change in the blood picture in Case 20 followed such substitution.

In Cases 20 and 21 the patients remained asymptomatic and showed no evidence of neurologic damage throughout the period of folic acid therapy. As described previously, the patient in Case 22 exhibited an explosive development of spinal cord disease during the eighth month of observation, despite a normal blood and bone marrow.

## DISCUSSION

### HEMATOLOGIC ASPECTS

*Induction of Remission* Orally administered folic acid induced hematologic remissions that were comparable in rate and completeness with those that would have been predicted in response to liver extract therapy. Reticulocyte responses to folic acid were less marked and subjective improvement was not so sudden in onset or so marked as usually follows exhibition of liver extract. Glossitis was not so rapidly or so completely relieved, and tended to recur in mild form during folic acid therapy.

*Maintenance Therapy* When folic acid was substituted for liver extract the majority of the patients showed a transitory but significant increase in blood levels and a decrease in mean corpuscular volume. This initial but unsustained improvement may have been due to the combined effect of folic acid and liver extract, since the latter is stored in the body and may continue to exert its hematopoietic effect for many months after its injection.<sup>4, 5</sup>

These observations might be taken to suggest that the patients had not received optimal doses of liver extract before substitution of folic acid, but most of them had been given large doses of liver extract without improvement in the blood picture. It seems unlikely that the improvement in response to folic acid occurred spontaneously, as a result of a nonspecific effect of folic acid or as a manifestation of the cyclic variations observed in pernicious anemia.<sup>6</sup>

With continuation of folic acid therapy alone there was usually a gradual fall in blood levels and the appearance of slight macrocytosis. This decrease usually became apparent after six to eight months of therapy, but in several cases it did not appear until the twelfth or fourteenth month of therapy. In most cases the blood returned to levels similar to those previously maintained with liver extract alone, but in several patients moderately anemic levels with definite macrocytosis appeared.

These findings may indicate that some factor in addition to folic acid is necessary for maintenance of a completely normal blood picture, and they suggest the possibility that a combination of orally administered folic acid and parenterally injected liver extract may be more effective than either substance alone.

*Dosage* All patients receiving folic acid in daily oral doses maintained blood levels at least comparable with those previously achieved with liver extract. During the period of our observations a daily oral dose of 1.25 mg of folic acid was as effective as a 15 mg dose in controlling hematologic manifestations. The patients who received the larger doses more frequently showed an initial improvement in

blood levels (table 2), but these higher levels were not better maintained with the larger than with the smaller doses

The injection of folic acid at monthly intervals did not maintain normal blood values. The rate of relapse in a patient who received injections of 30 mg once a month indicated that this amount of folic acid had no effect in maintaining remission. The simplicity and greater effectiveness of orally administered folic acid indicates that the oral route is the rational method of employing the substance.

#### NEUROLOGIC ASPECTS

Folic acid does not prevent the development or progression of subacute combined degeneration of the spinal cord in patients with pernicious anemia. In this series of 22 patients treated with folic acid, 7 (Cases 1, 8, 10, 14, 15, 17 and 22) developed neurologic relapse, 4 (Cases 3, 4, 12 and 13) showed progression of subacute combined degeneration, and 1 previously untreated case of severe subacute combined degeneration (Case 2) failed to improve during twenty-eight days of folic acid therapy. The blood levels were maintained within the range of normality in all but 1 (Case 1) of the patients who developed neurologic relapse, emphasizing the dissociation of the hematologic and neurologic aspects of the disease, and indicating that the effective hematopoietic factor is distinct from the factor necessary for the maintenance of normal central nervous system function.

Neurologic relapse occurred with considerable suddenness and progressed with great rapidity in several patients. This rapidity of progression was considerably greater than is usually observed in patients with untreated pernicious anemia. Apparently this also was true in similar cases reported by Meyer,<sup>7</sup> Heinle and Welch<sup>8</sup> and Vilter et al.<sup>9</sup>

We have continued daily oral administration of 15 mg of folic acid after instituting liver extract treatment in 5 patients (Cases 3, 4, 14, 17 and 22) in whom neurologic relapse developed during folic acid treatment. The period of observation with combined liver extract and folic acid treatment is still quite brief, but there has been progression of neurologic disease in 4 of these cases (Cases 3, 4, 14 and 17). Although improvement in gait and coordination occurred slowly in the fifth patient (Case 22) while folic acid was continued, vibration and position sense did not begin to show improvement until folic acid was omitted two months after the beginning of liver extract treatment.

Development or progression of subacute combined degeneration appeared to occur with the greatest frequency in patients receiving large daily doses of folic acid. Eight patients were receiving 10 or 15 mg daily by mouth when neurologic signs or symptoms first appeared, whereas only 3 developed neurologic disease when the daily dose was 5 mg or less. These observations are not conclusive because 4 of the cases on the 15 mg dosage had previously received smaller doses. These patients might have developed subacute combined degeneration on the lower dosage had it been continued, and the apparent precipitation of neurologic relapse when the dosage was increased may have been coincidental. We are impressed, however, by the fact that of 7 patients (Cases 9, 10, 11, 12, 18, 20 and 21) maintained on a low dosage (5 mg or less a day) for twelve months, only 2 (Cases 10 and 12) showed neurologic relapse. One of these (Case 12) had had long-standing

severe subacute combined degeneration and the progression of his disease was minimal. In marked contrast, of 6 patients treated with 10 or 15 mg of folic acid daily for twelve months (Cases 1, 3, 4, 5, 7 and 8), neurologic relapse occurred in 4 cases (Cases 1, 3, 4 and 8) within twelve months of the last injection of liver extract. The number of cases in each series is too small and the variation from patient to patient in susceptibility to subacute combined degeneration is too great to allow definite conclusions to be drawn from these observations, but it was quite certain that the patients who received large doses of folic acid developed neurologic relapse more frequently than did those who received small doses. It was of interest that of 12 reported cases of neurologic relapse occurring during folic acid therapy all received 10 mg or more of folic acid daily.<sup>7-10</sup> Heinle and Welch's patient apparently showed acceleration of subacute combined degeneration when the dosage of folic acid was increased to 100 mg daily. The neurologic signs apparently progressed even after liver extract was started, and did not regress until folic acid was discontinued. In contrast to our patients all of the reported cases were in hematologic relapse prior to the induction of folic acid therapy. All but 2 of our patients had been under intensive liver extract therapy before substitution of folic acid. It may be this difference, rather than the larger folic acid dosage, that accounts for the earlier development of subacute combined degeneration in the reported cases. The fact that the majority of our patients did not develop neurologic relapse until twelve months after the last injection of liver extract may have been due to residual stores of liver extract as a result of previous therapy.

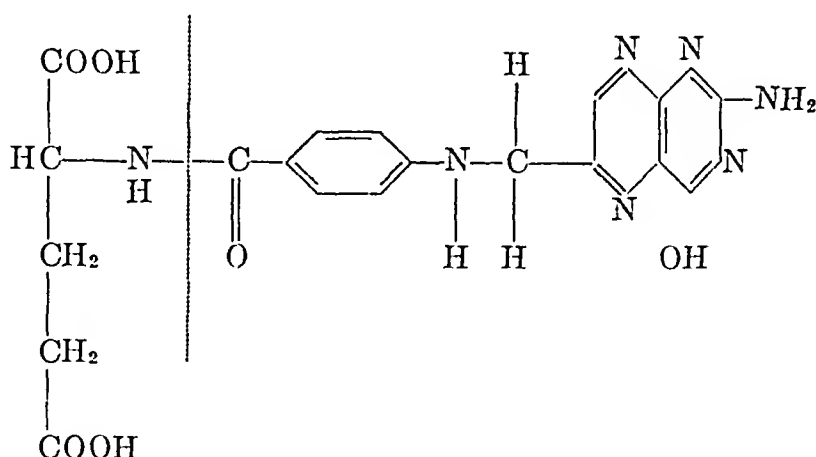
The failure of synthetic folic acid to prevent the development or induce the remission of subacute combined degeneration makes it certain that this substance is not the active principle responsible for maintenance of normal nervous system function in pernicious anemia. The possibility that folic acid in large doses actually may exert a deleterious effect on the central nervous system is suggested by three observations: the apparent greater tendency for the development of subacute combined degeneration in patients who received large doses of folic acid, the apparent acceleration of neurologic disease in some patients when folic acid dosage was increased, and the persistence or actual progression of neural disease following institution of liver extract therapy so long as folic acid treatment was continued.

Theoretically it seems possible that synthetic folic acid in large amounts may contribute to dysfunction of the central nervous system by interfering with the metabolism of l (+) glutamic acid by the central nervous system. The observations that suggest this theory are as follows. Quastel and Wheatley<sup>11</sup> found that l (+) glutamic acid could be metabolized by nerve tissue, and Krebs<sup>12</sup> showed that brain slices could utilize glutamic acid for the synthesis of glutamine. Weil-Malherbe<sup>13</sup> demonstrated that l (+) glutamic acid was the only amino acid that could be metabolized by central nervous system tissue. He observed that d (-) glutamic acid actually interfered with brain metabolism. The essential nature of l (+) glutamic acid in nerve function was further emphasized by Nachmansohn et al.,<sup>14</sup> who found that the enzyme system associated with the synthesis of acetylcholine in brain extracts, when inactivated by dialysis, could be reactivated by the addition of l (+) glutamic acid.

These fundamental observations establish the importance of l (+) glutamic acid



in nerve tissue metabolism. They also suggest its possible role in the formation of acetylcholine, a mediator of nervous impulses. Folic acid has the following structural formula



As indicated by the broken line, glutamic acid is one of the constituents of the folic acid molecule, and its position in the molecule suggests that it may be able to enter into competition with l (+) glutamic acid in tissue metabolism and interfere with nerve metabolism, and possibly with the formation of acetylcholine and the transmission of nervous impulses. If this interference does occur, it explains the greater frequency of neurologic relapse in patients receiving large doses of folic acid and the progression of neurologic disease in folic acid treated patients after the institution of liver extract therapy.

That folic acid is connected in some way with acetylcholine metabolism in clinical cases of pernicious anemia is indicated by Davis,<sup>15</sup> who reports that there is a marked *increase* in the serum acetylcholine of patients with untreated pernicious anemia, and that the administration of folic acid, liver extract or ventriculin produces a *decrease* in blood acetylcholine concentration.

We are now investigating the possibility that folic acid may actually contribute to dysfunction of the central nervous system by interfering with l (+) glutamic acid metabolism.

In view of recent reports that l (+) glutamic acid may be effective in the treatment of feeble-mindedness<sup>16</sup> and the possibility that folic acid may interfere with l (+) glutamic acid metabolism, it is of interest that 2 of our patients (Cases 3 and 4) who developed neural relapses were noted by their families to have had mental aberrations.

Whether or not synthetic folic acid in large doses actually interferes with central nervous system function is still to be determined. Clinical evidence proves beyond question that it is not effective in the treatment or prevention of subacute combined degeneration of the spinal cord. For this reason, it is our opinion that no patient with pernicious anemia should be treated with folic acid alone.

#### SUMMARY AND CONCLUSIONS

I. Twenty-one patients with pernicious anemia were maintained on synthetic folic acid (pteroylglutamic acid) therapy alone for periods ranging from eight to

seventeen months Satisfactory blood levels were maintained in all cases receiving daily oral doses of 1.25 to 15.0 mg Severe hematologic relapse occurred within six months in a case treated with monthly injections of 30 mg

2 Synthetic folic acid in oral doses of 15 mg daily induced satisfactory hematopoietic responses in 3 patients with pernicious anemia in severe relapse, but only slight hematopoietic response in a fourth patient with mild pernicious anemia but severe subacute combined degeneration of the spinal cord

3 Ten patients showed a significant improvement in blood values for a few months after substitution of folic acid for liver extract With one exception these subsided after six or more months to pre-folic acid levels comparable with those previously maintained with liver extract alone

4 These observations suggest that a combination of orally administered folic acid and parenterally injected liver extract may maintain a better hematologic status than either substance alone

5 A previously untreated patient with severe subacute combined degeneration of the spinal cord failed to show improvement in neural disease during twenty-eight days of folic acid therapy

6 Eleven patients developed, or showed progression of, subacute combined degeneration of the spinal cord during folic acid treatment Neurologic disease developed in most of these patients when the peripheral blood was normal

7 One patient showed an extremely explosive onset and rapid progression of neural disease The progression of the disease was rapid in 3 other cases

8 The institution of liver extract therapy in addition to folic acid in 5 patients who developed subacute combined degeneration during folic acid maintenance therapy failed to prevent progression of the disease in 4 cases, and only partially arrested the disease in the fifth, in which improvement occurred more rapidly when folic acid was discontinued

9 Subacute combined degeneration occurred with greater frequency in patients on large daily doses of folic acid than it did in patients with small or intermittent doses

10 The possibility is discussed that folic acid in large daily doses may actually precipitate or aggravate neurologic disease

11 It is suggested that folic acid may interfere with the metabolism of l(+)-glutamic acid in the central nervous system and possibly disturb the formation or function of acetylcholine

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## PERNICIOUS ANEMIA CAUSED BY DIPHYLLOBOTHRUM LATUM, IN THE LIGHT OF RECENT INVESTIGATIONS

By BERTEL VON BONSDORFF, M D

THE MACROCYTIC anemia caused by the broad fish tapeworm, *Diphyllobothrium latum*, has been the subject of extensive investigations in Finland, starting with the pioneer work of J W Runeberg (1887) and O Schauman (1894)

The fish tapeworm is extraordinarily common in the country. In certain provinces more than 90 per cent of the population is infested with the parasite. Of the total population of the country, which is about 4,000,000, one half to one million can be considered to be tapeworm carriers. The parasite is found in persons of all ages, even in children under 1 year of age.

For most of these carriers, the worm is a relatively innocent parasite, but in some, it causes a macrocytic anemia. According to earlier investigations (R Ehrstrom, 1926) the frequency of anemia was estimated to be 1 case in 5,000-10,000 worm carriers. But, if the blood of worm carriers is examined systematically, a far greater number of anemia cases can be shown. Among the conscripts of 20 years of age admitted to a military hospital in peace time, Seppa (1927) found 1 case of anemia in 659 worm carriers. G Totterman (1944), in a community strongly infested with tapeworm, noted, in 1942, 1 case of anemia in 136 carriers, and in 1943, he found the frequency to be 1 case of anemia in 383 carriers. In a war hospital, from 1941 to 1944, I found 96 instances of tapeworm anemia in about 11,000 medical cases. The number of worm carriers in this group, composed of men from 18 to 40 years of age, is not known.

The above figures give a good idea of the importance of the problem of tapeworm anemia in Finland. This anemia is a typical "pernicious anemia" with a characteristic blood picture and a megaloblastic bone marrow, agreeing in all respects with that found in "cryptogenetic" Addisonian anemia. A pernicious tapeworm anemia can also be brought to remission with liver preparations per os or parenterally, or with stomach preparations per os without removal of the worm.

In contrast to cryptogenetic pernicious anemia, tapeworm anemia occurs even in people under 20 years of age. In most cases there is an achlorhydria after the injection of histamine, though, especially in younger patients, the gastric juice often contains free hydrochloric acid and is secreted in the usual quantities. Signs of spinal cord involvement are less apparent than in cryptogenetic pernicious anemia.

That the illness is really due to the worm infestation is proved by the fact that complete remission takes place after the worm is expelled without any further need for antianemic treatment.

Since cryptogenetic pernicious anemia is not rare in Finland and worm infestation is common, it is natural that now and then both types of anemia should occur simultaneously in an individual. In such cases, of course, there is no remission after the worm cure. In fact, the anemia often becomes more severe.

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G Totterman (1944) says that he has encountered cases of mild macrocytic anemia in tapeworm carriers that did not respond to liver treatment but showed good remission after a worm cure. He assumes that the pathogenetic mechanism for this form of anemia is different from that for the true pernicious tapeworm anemia, which does respond to liver therapy.

On the other hand, there have been numerous reports in Finland of cases of hypochromic anemia in tapeworm carriers which were not cured by expelling the worm. On this point, conflicting information is still found in the literature.

The question which is of special interest for research is why do so few worm carriers have pernicious anemia? Or formulated in greater detail what are the factors which lead to the rise of worm anemia and why are these factors active in only a small number of cases?

Hitherto, these questions have not been satisfactorily answered. The discussion of them and the tapeworm anemia problem as a whole has been reported in detail by Birkeland (1932).

The present knowledge of the genesis of pernicious anemia suggests that in some way the worm interfered with the production or absorption of the antianemic factor or with the production of the components involved in its formation. This thought was first expressed by Saltzman (1935). On the basis of similar reasoning the pernicious anemia in connection with sprue, ventricular lesions, intestinal resections, etc., can, as is well known, be explained.

An investigation is here reported carried out on these lines with the object of explaining the pathogenesis of cases of pernicious anemia, most of them due to tapeworm infestation. The tests have been going on since 1937.

## 1 THE INTRINSIC FACTOR

a) *The course of remission after removal of the worm.* If the worm is expelled in the usual way (with 3-4.5 Gm. *Extractum filicis*) from hospital patients on an unrestricted diet, a blood remission begins shortly afterwards and continues until the blood picture has become normal. In a group of 7 cases, it was observed that the reticulocytosis began on the fourth to the sixth day after the worm was expelled in 6 of the patients and in 1 case, not until the eleventh day. The reticulocyte maximum was reached in the first 6 cases mentioned between the seventh and the tenth day after the worm cure, and in the seventh case on the thirteenth day. The maximum reticulocyte values varied between 7.4 and 23 per cent. In these cases, examination of the blood before *Extractum filicis* was given showed that the erythrocytes were between 1.2 and 2.1 million per cu. mm. of blood.

Apparently, after the worm cure, the reticulocytosis generally sets in a few days later than after a strong initial liver treatment. The maximum for reticulocyte values is on the average somewhat lower than in cases of pernicious anemia treated with liver, but on the other hand, the reticulocyte crisis lasts longer. The increase of the erythrocytes is generally about 100,000 per day, which is just as great as with adequate liver therapy.

It has been found by means of a sternal puncture that within 48 hours after the

worm cure, the megaloblastosis in the bone marrow gives way to a normoblastic type of regeneration

The most rational therapy in pernicious tapeworm anemia is the simple worm cure. Only in very severe anemia is it necessary to start treatment with liver injections and not expel the worm until after the blood picture has improved. In such cases, however, it is difficult to decide afterwards whether the pernicious anemia was caused by the worm or not. Only if the patient is very young and/or has free hydrochloric acid in the gastric contents can one be to some extent certain that the pernicious anemia was caused by the tapeworm.

The fact that the pernicious tapeworm anemia is completely cured after the worm is expelled can be explained in no other way than that the patients have access to all the substances required for the endogenous formation of the antianemic principle. Evidently, these substances are available directly after elimination of the worm, as indicated by the promptness with which the remission begins thereafter. If the food contains the extrinsic factor, this must imply above all that the intrinsic factor becomes at once available.

b) *Castle's test with gastric juice from patients with pernicious tapeworm anemia*  
Hernberg (1936-1941) has shown that the gastric juice in patients with pernicious tapeworm anemia, as well as in persons who have had it, contains intrinsic factor. Mixed with meat, such gastric juices produce a typical remission when given to patients with cryptogenetic pernicious anemia.

c) *In vitro experiments* The author has made some investigations of the proteolytic gastric enzyme active at neutral reaction according to the method given by Taylor et al. (1938), and has found (1940) that this enzyme occurs in pernicious tapeworm anemia as well as in cryptogenetic pernicious anemia, though in the latter cases the total amount of the gastric secretion is very much reduced. It has been suggested that this enzyme is identical with the intrinsic factor. My results have been confirmed by Helander (1945). Hernberg (1939) with Lasch's reaction has obtained similar results in the gastric juice from patients with pernicious tapeworm anemia.

The investigations here reported, all support the idea that the gastric juice of patients with pernicious tapeworm anemia contains intrinsic factor. In spite of this, and in spite of the fact that the amount of gastric juice is often normal, an anemia has arisen. Apparently the pernicious anemia in tapeworm carriers is not caused by cessation of the secretion of the intrinsic factor because of the presence of the worm. However, it is evident that some inhibition of the gastric juice secretion may occur in connection with pernicious tapeworm anemia, for in some cases free hydrochloric acid reappears in the gastric contents after the remission in patients who showed achlorhydria while the anemia was apparent.

The author has been unable to find any difference in the speed of remission after a worm cure in patients with achlorhydria and those with normal gastric secretion.

It seems possible that a decreased secretion of intrinsic factor may facilitate the occurrence of a pernicious anemia in connection with tapeworm infestation. It is well known that some people who have had a pernicious tapeworm anemia when young, have later fallen ill with a cryptogenetic form of the disease. In some

families many cases occur of both cryptogenetic anemia and pernicious anemia due to worm. The cause of this has been considered a special constitutional disposition for this type of anemia. It may be that this "disposition" consists in a deficient production of intrinsic factor.

## 2. THE EXTRINSIC FACTOR

a) *Clinical observations* Experience in Finland shows that worm carriers can have pernicious anemia even though their food contains a sufficient amount of protein of different kinds. Apparently, lack of extrinsic factor is not essential for the occurrence of pernicious tapeworm anemia (in contrast to some other conditions such as, for example, the nutritional tropical pernicious anemia). However, certain facts do support the idea that a relative deficiency in extrinsic factor can contribute to the rise of the disease. G. Totterman found in his material a higher frequency of pernicious tapeworm anemia in 1942 than in 1943. In the former year the food situation in our country was particularly serious, there was a special lack of proteins. The next year the situation had improved considerably. My own experience agrees with that of Totterman's. In some cases of worm anemia, I have seen a slight reticulocytosis and even a certain improvement of the erythrocyte count during the administration of meat. This was the case with soldiers who came for treatment directly from the front. Cramer (1922) observed pernicious tapeworm anemia at the same time in 3 sisters who, by reason of some mental peculiarity, lived like hermits and fed themselves with an extremely insufficient diet.

b) *The course of remission after removal of the worm in the absence of extrinsic factor* It is logical to assume that there will be no remission after the worm is expelled if the patient, shortly before and after the worm cure, has taken food containing no extrinsic factor. The correctness of this reasoning has been shown in a series of twelve tests. When the patients were admitted to the hospital they were placed on a basic diet as free from extrinsic factor as possible. After some days, they were given a worm cure. Very insignificant signs of blood regeneration, or none at all, were observed even after twelve to fifteen days. On the contrary, the blood picture often became progressively more abnormal. As soon as substances known or believed to contain extrinsic factor were added to the diet, a marked reticulocytosis began and the blood picture improved rapidly. It has been proved that this is true when meat, milk, Hammarsten's casein, pepton, brewer's yeast and concentrated yeast extract and, to a lesser degree, soy bean protein were added to the diet. These observations confirm Castle's theory that both intrinsic and extrinsic factor are necessary.

The method furnishes a means of testing substances for their content of extrinsic factor.

One practical conclusion is that, after worm cure, patients with worm anemia must be given a diet rich in proteins if a rapid remission is to be expected.

## 3. THE INTERACTION BETWEEN THE INTRINSIC AND EXTRINSIC FACTORS

Castle's test has not been previously carried out on patients with pernicious tapeworm anemia, yet this experiment is of great importance. It is conceivable that

the worm in the intestinal canal prevents the interaction between extrinsic and intrinsic factors and in this way gives rise to the pernicious anemia. If this is true, it would be expected that no remission would occur when a patient is given a mixture of meat and gastric juice. This would indicate that the worm has been able to destroy the effect of these substances supplied from outside in the same way as it prevents the body's own intrinsic factor from interacting with extrinsic factor in the patient's ordinary food. If a fresh mixture of gastric juice and meat proves to be ineffective while the same mixture incubated for six hours at  $37^{\circ}\text{C}$  does have an antianemic effect, the conclusion might be drawn that by means of the enzyme activity *in vitro*, some new substance is formed which the worm is unable to injure.

A series of 14 tests was carried out to clear up this question. Meat (150 Gm per day), or in some tests yeast extract, was used as the source of the extrinsic factor. The daily amount of gastric juice with which the meat or yeast was mixed was 150-175 ml. Each test period lasted eight days. During the first test period a nonincubated mixture was given.

In some cases of cryptogenetic pernicious anemia these tests produced a splendid remission. The effect was equally good whether the mixture was incubated previously or not. On the other hand, the test results were clearly negative in cases of pernicious tapeworm anemia. Neither fresh nor incubated mixtures of meat and gastric juice produced any remission. In some cases the identical gastric juice was used as in parallel tests with cryptogenetic pernicious anemia. The remission occurred only after the worm had been expelled.

In one case of pernicious tapeworm anemia, 100 ml of gastric juice was brought up daily after insulin stimulation, and was incubated with 150 Gm of meat for six hours, after which the mixture was administered to the patient. Not even in this way could any remission be produced.

These observations give strong support to the idea that the worm in the intestinal canal is capable of preventing interaction between the extrinsic and intrinsic factors and that such an inhibition can be deemed to be the reason for the pernicious anemia.

The fact that incubation does not involve an improvement of the antianemia effect of meat and gastric juice confirms the assumption that the antianemic principle cannot be formed *in vitro* but only *in vivo*. It is possible that the interaction between extrinsic and intrinsic factors takes place in the intestinal wall (Formix-nex, 1940). Perhaps this interaction is not a simple enzyme reaction.

#### 4 THE LIVER FACTOR

If the worm in pernicious tapeworm anemia is expelled and the formation of new antianemic factor is prevented by giving a diet free from extrinsic factor, then, as already stated, there is no blood remission. This shows that the liver must be deprived of its stock of antianemic factor, for if any were present, blood regeneration should take place after the anemia-producing worm had been removed, independently of the supply of intrinsic and extrinsic factors.

It is conceivable that the worm may destroy the antianemic factor at the place where it is assumed to be formed, *i.e.* in the intestine. Another possibility is that



the worm toxins absorbed from the intestine may destroy the entire quantity of antianemic liver factor available in the body, but this is an improbable theory

It is a priori somewhat improbable that the tapeworm can injure the liver factor, partly because it is rather stable and partly because we know that the administration of liver preparations both parenterally and per os quickly cures a pernicious tapeworm anemia

I have incubated ordinary injectable liver extracts together with worm in vitro at 37° C for some days and could not, at least in this way, prove any decrease of their antianemic effect

It appears, then, that the lack of the liver factor is not the result of destruction by the tapeworm itself nor by toxins from the worm

### 5 FOLIC ACID

I have treated 4 cases of pernicious tapeworm anemia with folic acid per os. An excellent remission was obtained in all cases with doses of 20-30 mg daily for 7-10 days, showing that folic acid also is not injured by the worm

### 6 THE ABSORPTION

The clinical picture in pernicious tapeworm anemia gives no reason to believe that the absorption in this disease is impaired. Carriers of *Diphyllobothrium latum* seldom suffer from severe intestinal disturbances. Worm carriers with and without anemia do not differ from each other in this respect. In no case are the conditions comparable with those in sprue, intestinal anastomoses, etc

The glucose tolerance test has been carried out in 4 cases of pernicious tapeworm anemia, both before the worm cure and after the blood had become normal. In all cases the blood sugar curve had a normal course both before and after the worm cure, thus it was not possible to show that there was any disturbance in the glucose absorption

### 7 EXPERIMENTAL FEEDING WITH TAPEWORM PREPARATION

The effect on the blood of giving worm preparations per os or parenterally has, of course, been studied in both animals and humans. T. W. Tallqvist (1907) experimented on himself in this way and G. Totterman (1938-1940) has published a large series of tests. Both have thought they saw a certain anemizing effect from the preparations they used. I am not convinced of the correctness of their conclusions for reasons stated in another publication

The problem has been attacked by attempting to answer the following questions (1) Is the antianemic effect of the mixture of gastric juice and meat nullified if worm is added? (2) Is the remission after the worm cure absent in worm anemia patients if the worm preparation is given per os?

The mixtures of gastric juice and meat (or yeast extract) were prepared in the same way as described earlier. The subjects were patients with untreated cryptogenetic pernicious anemia. First they were treated for eight days with gastric juice plus meat (or yeast extract) with the addition of a considerable amount of fresh or dried *Diphyllobothrium latum*. The remission was always splendid. During the

following period with gastric juice plus meat (or yeast extract) without the addition of worm, no new reticulocytosis was observed and the blood regeneration was no more rapid than during the first test period

In one case of worm anemia the patient, after the worm cure, was given worm powder per os in increasing amounts. In spite of this the blood improved in the usual way

In some tests the worm anemia patients were kept on a diet free from extrinsic factor, were given worm cure and then for eight days extrinsic factor in the form of yeast extract with the addition of worm powder. In spite of this addition the blood improved rapidly

In connection with these tests, worm powder was mixed with hog's stomach in order to investigate the possibility of loss of antianemic effect. In spite of the addition of powdered worms in two such tests the hog's stomach still had a marked antianemic effect

It was thus shown that addition of worm is unable to destroy the antianemic effect of mixtures of gastric juice and extrinsic factor, or of stomach preparations. Moreover, the presence of worm does not prevent the remission after the elimination of the worm in worm anemia. This fact has been interpreted to mean that the inhibition of the interaction between the intrinsic and extrinsic factors can be produced only by the living worm in its natural surroundings at the place where the interaction occurs

#### 8 INHIBITION IN VITRO OF THE PROTEOLYTIC ACTIVITY OF GASTRIC JUICE AT NEUTRAL REACTION

The gastric protease which is active at a pH range from 5 to 9 is greatly inhibited in its hydrolytic capacity in vitro after the addition of even relatively small amounts of *Diphyllbothrium latum*. The inhibitory substance is destroyed by heating to 80° C. for twenty minutes. It is not dialyzable and is not soluble in ether, nor in 98 per cent ethyl alcohol. It cannot be precipitated with 50 per cent alcohol, but can be precipitated quantitatively in 90 per cent alcohol.

The gastric protease in question has been assumed to be identical with the intrinsic factor, and the hydrolysis of casein in vitro has been considered as corresponding to the interaction between the intrinsic and extrinsic factors in vivo. It has not yet been possible to prove this assumption. As stated above it seems probable that such interaction cannot occur in vitro but only in the intestinal canal.

There is thus a discrepancy as follows: (a) The living worm in situ seems to inhibit the interaction between the extrinsic and intrinsic factors, (b) the administration per os of worm preparations does not inhibit this interaction, while again (c) the addition of worm in vitro inhibits the proteolytic activity of gastric juice at neutral reaction. It seems that this discrepancy cannot be explained until we have more detailed knowledge of the different substances here concerned. The exact chemical nature of the extrinsic factor, intrinsic factor and the tapeworm toxin are as yet unknown.

## 9 THE LOCALIZATION OF THE TAPE WORM IN THE INTESTINAL CANAL

Presumably the worm cannot inhibit the reaction between extrinsic and intrinsic factor unless the worm is present at the place where the interaction occurs. Now the question arises: Where in the intestinal canal is the worm to be found? Very uncertain information on this point is available. Experience from operations and autopsy in general indicate that the worm has been chiefly observed in the ileum, but there are no systematic observations of this fact. Sometimes it happens that *Diphyllobothrium latum* is vomited, which shows that at least occasionally it can be very high up in the intestine. To investigate this question, the author made a series of intestinal intubations. As the *Diphyllobothrium latum* produces large quantities of eggs it was relatively easy to determine at what distance from the

TABLE 1 — Distance from Mouth (cm.) where Ova and/or Proglottids of *Diphyllobothrium Latum* Were Found

Group 1 No anemia	Group 2 Nonpernicious anemia	Group 3 Pernicious tapeworm anemia, manifest	Group 4 Pernicious tapeworm anemia in spontaneous remission
235	334	135	320
235	180	120	240
230	140	120	205
180		120	
180		115	
180		115	
165		110	
150		105	
145		105	
130		95	

mouth the first eggs could be aspirated. In many cases small pieces of the worm itself were aspirated at the same time. Of course, it is not possible to calculate in this way the highest point in the intestine where the worm is attached. Although no eggs are produced from the highest segments of the worm, yet I have been convinced that results can be obtained which allow comparison between different cases.

The intubations were carried out on 26 worm carriers who were divided into 4 groups as appears in table 1, in which the results are also summarized.

The results show that in manifest pernicious anemia the worm is found higher up in the intestine than otherwise. Perhaps one can imagine that in that region it is better able to interfere with the interaction between extrinsic and intrinsic factors. How high up in the intestine the worm must be for it to inhibit this reaction is difficult to determine. My results favor the opinion that a 'critical limit' lies 140-150 cm. from the mouth, which ought to be about the borderline between the jejunum and the ileum.

## 10 AN ATTEMPT TO EXPLAIN THE PATHOGENESIS OF THE PERNICIOUS TAPEWORM ANEMIA

As described above, it appears to be possible that the *Diphyllobothrium latum* causes pernicious anemia by inhibiting the interaction between extrinsic and intrinsic

sic factors, but that this reaction can occur only if the worm is sufficiently high in the intestine. However, as stated previously, there is reason to presume that the amount of extrinsic factor in the food and of intrinsic factor in the gastrointestinal canal is also of some importance. Thus whether anemia occurs or not would depend on a definite correlation among these three determinants: the amount of extrinsic factor, the amount of intrinsic factor, and the worm's high or low position in the intestine.

Finally a time factor must also be taken into consideration. The formation of antianemic factor must have been inhibited for such a long time that the liver is wholly deprived of it. Only then is there reason to expect that the anemia will manifest itself.

It must be emphasized that in the great majority of cases of pernicious tapeworm anemia, there is no basis for the assumption that either a defective diet or a hereditary disposition are to be reckoned with as co-operating causes. Most tapeworm anemia patients do *not* fall ill later with a cryptogenetic anemia. I can, therefore, not confirm the correctness of Birkeland's conclusion that "it seems appropriate to classify surviving patients with *Diphyllobothrium* anemia as suffering from abortive forms of genuine pernicious anemia." My view of the problem is that in principle any worm carrier whosoever can get a pernicious tapeworm anemia if only the worm—*ceteris paribus*—is high enough up in the intestine. If the worm is expelled a complete restitution can follow.

The theory I have formulated explains—in my opinion—the following circumstances which have been specially put forward by Saltzman (1924) and which have hitherto been difficult to interpret:

a) *A person can carry *Diphyllobothrium latum* for many years before he falls ill with pernicious anemia.* The explanation of this can be that the worm, for one reason or another, has invaded the upper parts of the intestine, sometimes possibly as a reinfection.

b) *A person who has had pernicious worm anemia and becomes well after the worm is expelled does not necessarily get anemia if he is again infested with worm.* At the reinfection it may happen that the worm is only in the lowest parts of the intestine.

c) *A worm expelled from an anemia patient is often disintegrating and discolored.* Sometimes no worm at all is seen in the feces. According to my theory, this disintegration can be due to the fact that the worm, being higher up in the intestine has had a longer distance to go before it was expelled. During its passage through the intestine it has had to undergo a strong autolytic decomposition and is also affected more by the digestive enzymes than if it had been in the lower part of the small intestine and only had to pass through the colon, where the enzymes are less active.

d) *The amount of worm is not in correlation with the occurrence of anemia.* A small amount of worm can cause anemia if it is sufficiently high up in the intestine, while a large amount does not necessarily do so if it is collected in the lower part of the small intestine. Yet cases with very large amounts of worm, 80–100 M and more, are often accompanied by anemia. In such cases it can be imagined that the worm, because of its great volume, has been forced upwards towards the jejunum.

e) *Spontaneous remission* with a return to normal blood values are not rare in worm

anemia The explanation of this can be that the worm had deserted the upper parts of the small intestine and wandered down towards the ileum In my 3 cases of this type the worm was found just as low in the intestine as in nonanemic worm carriers (cf table 1)

f) *Remission after an incomplete worm cure can also occur* A filicin cure can fail in such a way that only a small amount of the worm, or none at all is expelled, and after the cure worm eggs can still be seen in the feces In spite of this, the blood improves At a later worm cure—after the blood picture has become normal—a considerable amount of worm is often removed In such cases—according to my idea—the worm at the first 'unsuccessful' cure was driven from the upper part of the intestine but remained in the lower part where it was no longer able to exercise its anemia-producing effect

This idea was confirmed by the following test In one case of manifest pernicious worm anemia the eggs were found 115 cm from the mouth Through the intestinal tube 2 Gm filicin emulsion were instilled The worm was not expelled and the feces continued to contain worm egg, but they could not be demonstrated as present at the former depth (115 cm) A few days after the filicin cure a marked reticulocytosis began and the blood picture improved rapidly The tube was then allowed to glide farther in, and worm eggs were not found till 200 cm from the mouth, that is, far down in the ileum Following another treatment with filicin, 31 M of ordinary looking *Diphyllobothrium latum* were expelled

#### COMMENT

According to one earlier theory, the occurrence of worm anemia may be due to a change in the character of the parasite, possibly an abnormal disintegration of the worm in the intestine, but I have been unable to find any signs of such a disintegration The worm segments which I sometimes aspirated at intubation from worm anemia patients have been very motile and of ordinary appearance According to other theories, the cause of the anemia lay in the host These theories have presumed a varying permeability of the intestinal wall to the worm toxin, a special, individual susceptibility of the hemopoietic organs to it, or an allergic preparedness, G Tötterman has classed the pernicious tapeworm anemia with the malignant granulocytopenia due to the use of amidopyrine Apart from the fact that I find the experimental basis of these theories defective, none of them seem to me to explain the worm anemia problem satisfactorily It appears artificial to conceive of the macrocytic anemia with its megaloblastosis as an allergic reaction Another fact that tells against the toxic and allerge-toxic theories is that the anemia can be cured with liver or stomach preparations without expulsion of the worm Again, on the basis of them, it is difficult to explain why no remission occurs after the worm cure unless extrinsic factor is available The circumstances listed under Section 10 (a-f) above are also not easy to explain

There is no doubt that *Diphyllobothrium latum* contains a powerful poison If one handles fresh worm with unprotected hands, the skin is greatly irritated If one places a small amount of dried and pulverized worm on the tongue, there is a feeling of burning The inhalation of worm powder has been proved to produce nausea,

fever, rhinitis, asthmatic cough and eosinophilia in the blood. A worm carrier often suffers from giddiness, various nervous manifestations and nausea, has eosinophilia (which may or may not be absent in anemia cases) and shows serological changes. Like the macrocytic, nonpernicious anemia described by G. Totterman, these phenomena can be considered as the expression of the effects of toxic activity, resembling those which condition the rise of pernicious anemia. Thus, according to my idea, the inhibition of the interaction between extrinsic and intrinsic factors is only *one* expression of the worm's toxicity.

Tests on animals with worm preparation injections also bear witness to the toxicity of the worm. Among other things, it appears to contain a hemolytic toxin. Yet it has not been possible with these tests to produce an anemia which directly corresponds to the Addisonian anemia in man.

Much confusion in the discussions could, I believe, be avoided if the ability of the worm to produce pernicious anemia was consistently kept separate from its other toxic properties.

Certain details in the tapeworm anemia problem still await solution. Like the cryptogenetic pernicious anemia, the pernicious tapeworm anemia shows definite variations in its seasonal distribution. It is most usual during the period from March to August. Illustrative curves with which my own experience agrees are to be found in Birkeland's monograph. It is at present impossible to decide to what extent these seasonal variations depend on circumstances in the patient himself on light conditions, on the contents in the diet of protein, folic acid (as suggested by Waldenstrom), or other substances. A racial factor (in connection with pigment metabolism) must also be taken into consideration in both forms of pernicious anemia.

The chemical nature of the worm's toxin is still unknown. If that could be determined, it might be possible to get a better idea of the process by which the poison interferes with the interaction between the extrinsic and the intrinsic factors.

I have tried to show here that the investigation of pernicious worm anemia has not only a local interest but can also contribute to the elucidation of the whole great question of the macrocytic and megaloblastic anemias which respond to liver treatment and which are termed "pernicious."

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# THE STUDY OF THE MYELOGRAM (BONE MARROW PUNCTURE) IN PERNICIOUS ANEMIA AND THE PROBLEM OF THE MEGALOBLAST

By JACQUES MALLARMÉ, M D

OF ALL the recent advances in medicine, the conquest of pernicious anemia is one that should make pathologists proud. Twenty-five years ago, Biermer's anemia was a pernicious anemia, often fatal and which could not be cured by any treatment. It is fair to acknowledge the brilliant work of Minot and Murphy, who conceived the idea of applying the liver treatment inaugurated by Whipple, to pernicious anemia. The results were such that since then, patients with pernicious anemia do not die of the disease any more. Simultaneously, hepatotherapy has provided a convincing argument in favor of the specificity of the disease. Confusion with other severe types of anemia has been avoided. In addition to hepatotherapy other arguments have supported the concept of pernicious anemia as an autonomous disease, entirely distinct from other types of anemia. Among these arguments, one of the most recent is based on the study of the myelogram in pernicious anemia.

The cytology of the bone marrow was already known before the use of sternal punctures. However, postmortem examinations are vitiated by the onset of cadaveric changes in the tissues. Therefore, the sternal biopsy is indispensable to an understanding of the bone marrow. This is why the technic of bone marrow biopsy, introduced in 1929 by Arinkin, provided a great impetus to the development of hematology. This applies particularly to the study of pernicious anemia.

Sternal puncture is a simple procedure carried out with a special needle containing a stilet, which penetrates easily through the anterior table of the sternum, local anesthesia is not necessary. The bone marrow is aspirated in very small quantities if one wants to have a rich myelogram. Reading of the myelogram is easy if the technic of preparation is adequate: spreading—compression between two slides of a very small quantity of tissue, preferably small fragments of bone marrow—double staining by May-Grunwald-Giemsa for one-half hour. The water used should have a neutral pH.

Naegeli first described the erythroblasts which characterize pernicious anemia. He called them *megaloblasts* and this term was responsible for much of the confusion that prevailed thereafter. This confusion arose from the fact that Ehrlich and also Jolly gave the name 'megaloblast' to certain types of very young erythroblasts which are basophilic and are found in any normal marrow. These are not the megaloblasts which are found in pernicious anemia. These normal erythroblasts are also called pronormoblasts, basophil normoblasts or Naegeli's normoblasts.

The megaloblasts found in pernicious anemia represent a special lineage of erythroblasts, ultimately ending up in a special type of erythrocyte, the megalocytes, and having a characteristic morphology at each stage of the cytologic development.



As seen in the bone marrow smear, the megaloblasts of pernicious anemia have a high optical density. The youngest forms arising from the reticulum are the promegaloblasts (called "erythrogonos" by certain authors). They have a histioblastic appearance with a grayish blue protoplasm, more or less spread out, not sharply limited, with pseudopods and sometimes a protoplasmic connection with another promegaloblast. The nucleus is enormous and the chromatin shows a fine lacy structure with numerous bluish nucleoli.

Following the promegaloblasts come the basophilic megaloblasts which are frequently seen. These cells are large with a big round nucleus centrally placed. The chromatin has a fine skeinlike structure and is transparent. The protoplasm is very basophilic, has a variable thickness and is usually quite wide. In some cases, the basophilic megaloblast shows early eosinophilic granules.

As maturation progresses, the cellular anarchy becomes more manifest and the aspect of the megaloblast diverges more and more from that of the normoblast. The nucleus remains large, with a fine transparent chromatin, which in places condenses into pearl-like or blocklike structure. The peripheral protoplasm becomes lighter in an irregular fashion and its maturation may lag behind or, more often, precede that of the nucleus.

The fourth stage of the development is that of polychromatophilic megaloblast. The asynchronism between nucleus and protoplasm becomes manifest. A big nucleus may be found in conjunction with a small cytoplasm or a small nucleus in conjunction with a large cytoplasm. The cytoplasm shows vivid colors, purple or greenish with heterogeneous areas of variable shapes. The nucleus still has a partly reticulated structure and may undergo amitotic division or fragmentation which produces an aspect of a petalled flower. The nucleus is still young as shown by its transparent and pearl-like aspect. But the tendency toward the formation of fragments of the nucleus classifies the cell as an old type of cell. The nuclear fragments appearing early in the cell are the future Jolly's bodies.

The orthochromic megaloblasts have an orange color but may contain basophilic remnants in the form of basophilic areas or granules as in lead poisoning. The nucleus may be round and regular as the nucleus of the orthochromic normoblast, but more often it is reniform or dumbbell-shaped or has the shape of a clover leaf. The orthochromic megaloblasts vary in size and some may be rather small. When they lose their nucleus they become remnants, Cabot's rings, and Jolly's bodies. The megaloblasts in pernicious anemia often show atypical mitotic figures at all phases.

In summary, at all stages of the evolution, the megaloblast is an abnormal dystrophic erythroblast resulting in the creation of a large erythrocyte. This explains the anisocytosis, poikilocytosis and megalocytosis which are the expression of a cellular dystrophy and not of a cellular immaturity since most cells observed in the blood smear of a case of pernicious anemia are completely mature cells.

After a long period of discussion, hematologists agree among themselves that the megaloblastosis belongs specifically to pernicious anemia. I, personally, do not think that megaloblasts are observed in any anemia except pernicious anemia, provided one adheres to the definition of the megaloblast as I have given it, and one does not consider the megaloblast as a very young nucleated basophilic erythrocyte.

Every time I have been invited to see so called megaloblasts in the myelograms from patients having blood disease but no pernicious anemia, I have recognized that these were not megaloblasts. While the megaloblast is specific for pernicious anemia, there are also normoblasts and their proportion to the number of megaloblasts is variable. They are seen in early cases of pernicious anemia. Also they completely and rapidly replace the megaloblasts as soon as hepatotherapy is instituted. This fact permits one to conclude that the megaloblastosis is conditioned by a deficiency of the hemopoietic factor of maturation of red cells which is provided by the liver.

The generally accepted opinion, proposed by Naegeli, by Ferrata, and most hematologists is that the megaloblast represents a revival of the embryonic erythrocyte of the first generation. There is a certain morphologic analogy between the megaloblast and the nucleated erythrocyte of the fetus. One arrives therefore at the conception, as already advanced by Dameshek and Wilkinson and Israels, that there are two types of hemopoiesis: the normoblastic or adult type and the megaloblastic or embryonic type. Their appearance or disappearance depends on the factor of Whipple: if the maturation factor is deficient, the fetal type of erythroblasts appears in the bone marrow but if the factor is administered, the normal type of erythroblasts replaces the megaloblasts.

My interpretation is a little different from what precedes. The megaloblast does not appear as the result of a substitution of two erythroblastic lineages, because such a substitution is never observed. Rather than a substitution, a real transformation occurs which changes the normal erythroblast into a megaloblast: this morphologic transformation is caused by a deficiency of the maturation factor. In other words the megaloblast is a normoblast suffering from a nutritional deficiency.

This interpretation explains several particularities of the disease: first of all, the megaloblast may exhibit morphologic monstrosities in a varying degree according to the degree of the deficiency. This is analogous to what is seen in dystrophies caused by endocrine or vitamin deficiencies. In very advanced cases of pernicious anemia, the megaloblasts are typical and numerous. In incipient cases, the erythroblasts are not very much different from normoblasts and there are intermediary forms between normoblasts and megaloblasts. Finally when the treatment by liver injections is instituted, there is a rapid transformation of the megaloblasts into normoblasts.

The metaplasia from normoblast to megaloblast, or vice versa, always affects the young forms of the series: proerythroblasts and basophilic erythroblasts. This is why in cases of pernicious anemia in relapse the young basophilic cells are megaloblastic while the older cells are normoblastic. As soon as the liver treatment is instituted, the myelogram shows a normoblastic transformation of the basophilic erythroblasts without change in the polychromatophilic and orthochromic megaloblasts.

Another proof of the existence of a nutritional dystrophy in pernicious anemia is provided by the aspect of the granulocytes and megakaryocytes of the bone marrow.

The granulocytes show morphologic changes: the myelocytes are very large and

pale looking with enormous nuclei. The metamyelocytes have a ribbon-shaped nucleus with pseudopods. The polymorphonuclears have a polysegmented nucleus (up to 15 segments) giving the appearance of a knotted chord.

The megakaryocytes also have a polysegmented nucleus. All the cells formed in the bone marrow are affected in pernicious anemia. *Biermer's disease is a dystrophic myelosis, affecting the bone marrow as a whole, and producing anemia, neutropenia and thrombocytopenia.* The morphologic changes resulting from the dystrophy are specific and constitute the basis for the diagnosis.

From a scientific point of view, the myelogram in pernicious anemia is very interesting because it constitutes an instance of a cytologic dystrophy which can be reversed by the administration of liver, of Castle's factor, of folic acid.

One cannot help being struck by the analogy between the cytologic dystrophy of pernicious anemia and certain cellular alterations of malignant neoplastic tissues. In both cases there is the same excessive proliferation, the same cellular monstrosity, the same cytoplasmic-nuclear asynchronism, the same young aspect of the nucleus, same abnormal mitotic or amitotic cellular division.

On the other hand, hepatotherapy or folic acid treatment are very similar to vitamin therapy.

At the present time, therefore, one can say without exaggerating too much that pernicious anemia is a nutritional deficiency and a disease rather akin to cancer, in which the abnormal cellular proliferation is corrected by a chemically defined organic substance.

## REFRACTORY MEGALOBLASTIC ANEMIA

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THE TERM "refractory anemia" was introduced by Bomford and Rhoads (1941) for anemias of a wide variety of types that were refractory either temporarily or permanently to hematinic therapy. In 1943 Davidson, Davis and Innes published a series of papers entitled "Studies in Refractory Anaemia" which dealt with the problem of classification on the basis of examination of the bone marrow by sternal puncture and divided the anemias refractory to liver extracts into two main groups, namely (1) refractory anemias with hypocellular normoblastic marrows, and (2) refractory anemias with hypercellular megaloblastic marrows. Of particular significance was their finding that the prognosis was vastly different in the two groups. Thus, of 16 patients in Group 1, 11 died of progressive anemia within a few months, while of 16 cases in Group 2 all eventually made a complete recovery. Intensive treatment with large amounts of liver extract supplemented with iron and vitamins and repeated blood transfusions was required for long periods if such satisfactory results were to be obtained. The long period of illness during which life was continuously in danger indicated the need for some therapeutic agent which would cause a prompt remission comparable to that obtained with parenteral liver therapy in the relapse stage of Addisonian pernicious anemia.

In this paper the term refractory megaloblastic anemia is confined to cases of megaloblastic anemia which failed to respond hematologically and clinically to the parenteral administration of an amount of liver extract which has been proved to produce an optimal response in cases of Addisonian pernicious anemia. The test preparation employed was Anahaemin, marketed by British Drug Houses Ltd, which has been found by the writer to be potent in a dose of 2 cc when administered parenterally in a large number of cases studied during the past ten years. Every patient with refractory megaloblastic anemia received at least twice this dose after admission to hospital. In addition many cases had received large amounts of potent liver extracts prior to being referred to us for investigation of their failure to respond. Since infections, intoxications and advanced arteriosclerosis are known to inhibit or delay the response to parenteral liver therapy, patients exhibiting any of these complications were not included in the group of refractory megaloblastic anemia discussed below.

For many years the writer has suggested that chemical purification of liver extracts for parenteral use removes some essential factor which is necessary for the restoration of normal blood formation in certain cases of megaloblastic anemia which have failed to respond to potent liver extract given parenterally. The following case history of a patient seen by the writer nearly fifteen years ago illustrates this problem very clearly.

The patient was a middle-aged business man who had worked in India for many years and had always been in good health until one year before the present illness. His case notes from Calcutta indicated that

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a year previously he had had an attack of dysentery from which he apparently recovered completely. A few months later he began to feel tired and breathless on exertion. His tongue became sore and his bowels loose. The report from a medical specialist in Calcutta indicated that he had a moderate degree of macrocytic anemia and free hydrochloric acid was present in the gastric juice. Despite all treatment, he continued to lose weight and strength rapidly and was sent home to Scotland for investigation of the cause of his illness. When I first saw the patient he was extremely emaciated having lost 6 stone in weight during the previous twelve months. He was passing pale, greasy, bulky stools and the blood picture was typical of Addisonian pernicious anemia. His red cells numbered 1 M and his Hb 25 per cent. A histamine-fast achlorhydria was present. He was diagnosed as suffering from tropical sprue and given a low fat diet supplemented by vitamins and iron. Parenteral treatment with the liver extract Campolon was started. This resulted in a rise of reticulocytes to 15 per cent but no subsequent increase in red cells or Hb occurred. The patient was desperately ill and life had to be maintained by blood transfusions. Parenteral liver therapy was continued but was totally ineffective. The patient's diet was then changed to a high protein diet containing 150 Gm. of protein daily in the form of meat and liver by mouth. Within a week a remarkable improvement in his general condition and hematologic state occurred. Within three months the patient gained nearly 4 stone in weight and his blood count and blood picture were restored to normal. Of particular interest was the finding that free hydrochloric acid was again present in his gastric juice. Contact was kept with this patient for many years and it was found that the complete clinical and hematologic remission was maintained.

This case represents a perfect example of a refractory megaloblastic anemia associated with the sprue syndrome which failed to respond to large quantities of crude potent liver extract given parenterally and showed a dramatic improvement when given liver by mouth.

During the next ten years I occasionally encountered patients with the classic pernicious anemia blood picture who were refractory to parenteral liver therapy but who responded to liver given orally. The problem of refractoriness was brought into prominence during an investigation which was conducted in Edinburgh into cases of pernicious anemia of pregnancy. In this group of megaloblastic anemias we found that refractoriness to potent liver extracts given parenterally is not uncommon. The results of the investigation were published in 1942 (Davidson, Davis and Innes). Of 16 cases with a classic megaloblastic marrow 10 were refractory to liver extracts given parenterally. Shortly after this investigation our attention was attracted to the megaloblastic anemias associated with the sprue syndrome (tropical sprue and idiopathic steatorrhea), and here again we found patients who were either completely or partially refractory to potent liver extracts given parenterally. In addition to cases of refractory megaloblastic anemia associated with pregnancy, and the puerperium and the sprue syndrome we also encountered cases of refractory anemia whose etiology was completely obscure, and to this group we gave the name 'idiopathic refractory megaloblastic anemia' and it is with this group that this paper is particularly concerned.

This short introductory note regarding our clinical investigations into refractory megaloblastic anemias over many years is given with the object of indicating why we desired to find a therapeutic agent which would be effective and why we believed that this product could be produced from liver which had not been submitted to a process of chemical purification for parenteral therapy.

The first step in this investigation consisted of predigesting liver with the enzyme papain at a pH of 5.6, thus avoiding the danger of destruction of active principles

by exposure to acid or alkaline conditions. The product obtained was a light brown powder completely soluble in water. The name "proteolysed liver" was selected for descriptive purposes. Since the walls of the liver cells had been completely disrupted it appeared likely that a high proportion of water soluble constituents would be liberated and hence retained in the final product, and that other active principles present as a protein complex would be set free and so be rendered available for immediate absorption. Clinical tests made with a 70 per cent alcohol soluble fraction of liver before and after digestion with papain supported this conclusion.

It was estimated that 1 oz. of "proteolysed liver" was derived from 6 oz. of raw "wet" liver. The material which has since been marketed under the trade name "Hepamino" was first tested on 5 cases of classic Addisonian pernicious anemia and produced a dramatic response in all instances in a daily dose of  $\frac{1}{4}$  to  $\frac{1}{2}$  oz. A report on the method of preparation and its clinical trial was published in 1943 (Davis, Davidson, Riding and Shaw). During the next two years work was extended to testing the preparation in cases of refractory megaloblastic anemia. Thus, in 1944, we described the remarkable results produced in 4 cases of idiopathic megaloblastic anemia and in 1 case of refractory megaloblastic anemia of pregnancy (Davis and Davidson). We also noted its therapeutic failure in cases of macrocytic anemia with a normoblastic marrow.

We suggested as a provisional hypothesis that "While failure of maturation of the megaloblasts in the great majority of megaloblastic anemias is due to deficiency of the liver principle of Castle present in fractionated liver extracts, in refractory megaloblastic anemias it results from an additional deficiency consequent on a failure in production or absorption of some unknown factor which is present in adequate amount and assimilable form in proteolysed liver and presumably also in whole liver." In the same paper we discussed the possible nature of this factor and came to the conclusion that it was unlikely to be a mineral, an amino acid, or any of the vitamins available at that time for clinical use. We suspected that it might be folic acid or biotin, both of which were known to be present in considerable quantities in liver. From our assessment of the position we felt that folic acid was most likely to be the missing factor and accordingly, in 1944, we wrote to Dr. Riding of Evans Medical Supplies Ltd., asking him to make a preparation of folic acid for clinical trial in refractory megaloblastic anemias. The folic acid fraction sent to us for this purpose consisted of material precipitated by 70 per cent alcohol from a watery extract of liver. This fraction is discarded in the manufacture of parenteral liver extracts as it has been repeatedly shown to be impotent therapeutically. Nevertheless, it is in this fraction that most of the folic acid in liver is stated to occur, as determined by biologic assay. As was to be expected from previous clinical experience, this fraction was found to be impotent when fed in daily doses of 1 oz. to patients with pernicious anemia. Unfortunately at that time no suitable case of refractory megaloblastic anemia was available on which to try the extract. These results suggested that if folic acid was present in the nonproteolysed 70 per cent alcoholic precipitate of liver, it existed in some conjugated form which could not be utilized by a patient with pernicious anemia or, alternatively, that

the content of folic acid in the test dose of extract was insufficient. The next step taken was to submit the 70 per cent alcoholic precipitate of liver to papain digestion and see whether this would lead to the liberation of some hematinic factor which was not available in the nonproteolysed 70 per cent alcoholic precipitate. Three cases of Addisonian pernicious anemia were treated with this proteolysed fraction. Of these, 1 case responded moderately well and 2 failed to show any response. The single success achieved suggested that as a result of enzymic digestion some potent material had been liberated but that the amount so liberated was insufficient to produce satisfactory results. From an assay of folic acid in proteolysed liver carried out at a later date, this supposition was almost certainly correct. Accordingly we decided to obtain a more potent source of folic acid and were in the process of investigating measures to achieve this object when the synthesis of folic acid was announced by Angier et al (1945) and its clinical and hematologic effects in megaloblastic anemias were published by Spies et al (1945). Through the courtesy of Messrs Lederle and Dr Spies, we were fortunate in obtaining adequate supplies of folic acid and have thus been able to confirm the observations made by Spies and other workers in America in regard to its effectiveness in all forms of megaloblastic anemia (Davidson and Girdwood, 1946, 1947). Of particular interest to us was the determination of the smallest dose of synthetic folic acid which would produce a hematologic response in pernicious anemia as this was obviously a matter closely related to the problem of what constituent in proteolysed liver was responsible for its therapeutic activity in megaloblastic anemias refractory to potent parenteral liver extracts. In this connection we should mention that of 5 cases of pernicious anemia treated with 2.5 mg of folic acid daily all responded satisfactorily. Of 5 cases given 1 mg daily 1 gave an excellent response, 2 a moderate response and 2 gave no response. The last 2 cases subsequently responded well to a daily dose of 2.5 and 5.0 mg respectively. The problem of the minimal effective dose of folic acid is referred to again in the discussion.

#### IDIOPATHIC REFRACTORY MEGALOBlastic ANEMIA

During the past six years more than 450 cases of macrocytic anemia have been submitted to a full clinical and hematologic examination including sternal biopsy in the wards and blood clinics under my charge. Approximately 75 cases out of this group were found to be cases of macrocytic anemia with a normoblastic marrow and need not be considered in this paper. They included many examples of the sprue syndrome and cases of chronic hepatitis, aplastic and hypoplastic anemia, macrocytic hemolytic anemia and aleukemic leukemia. Three hundred and fourteen cases were diagnosed as classical pernicious anemia and all responded satisfactorily to parenteral liver therapy, proteolysed liver or folic acid. In addition, parenteral liver therapy was effective in 12 cases of megaloblastic anemia associated with pregnancy and the sprue syndrome. Lastly there were 59 cases of megaloblastic anemia refractory to potent parenteral liver extracts. Since a description has been given in our previous publications of cases associated with pregnancy and the puerperium and with the sprue syndrome it has been decided merely to illustrate representative cases of these groups with graphs showing the effect of treatment.

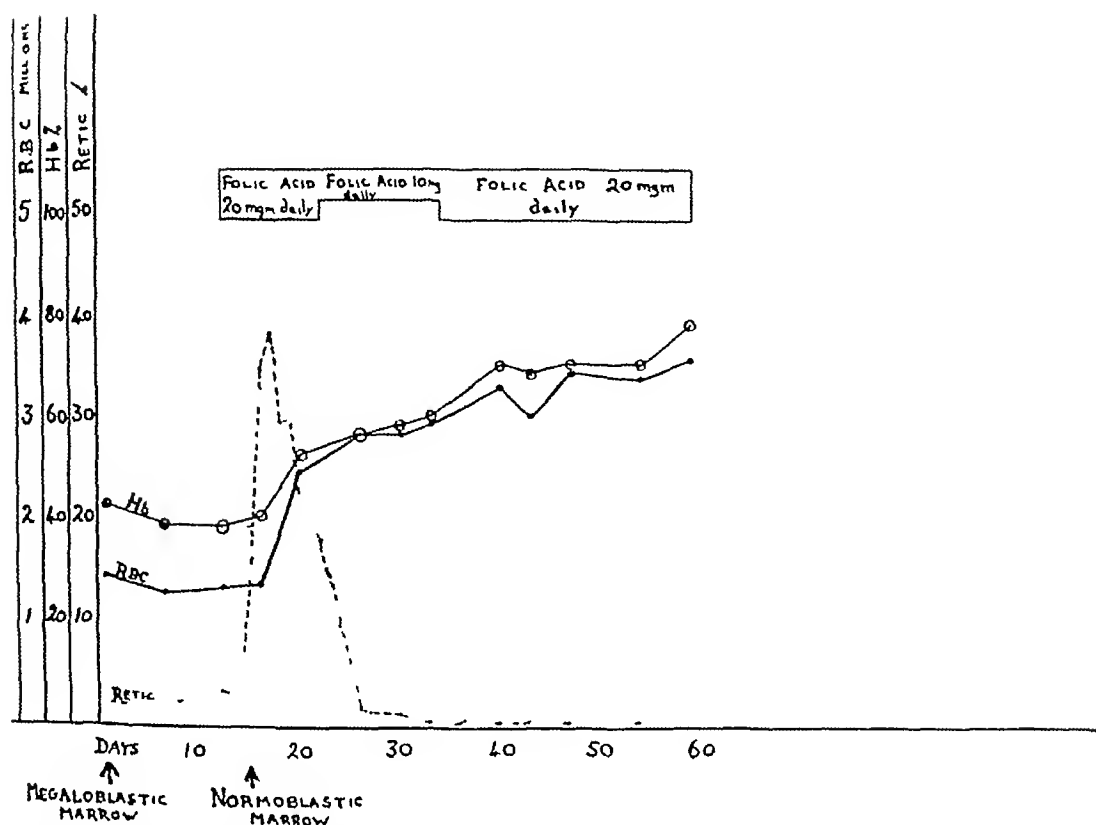


FIG 1 A woman, aged 37, with refractory megaloblastic anemia associated with idiopathic steatorrhea. Response to folic acid (See Case History 2.)

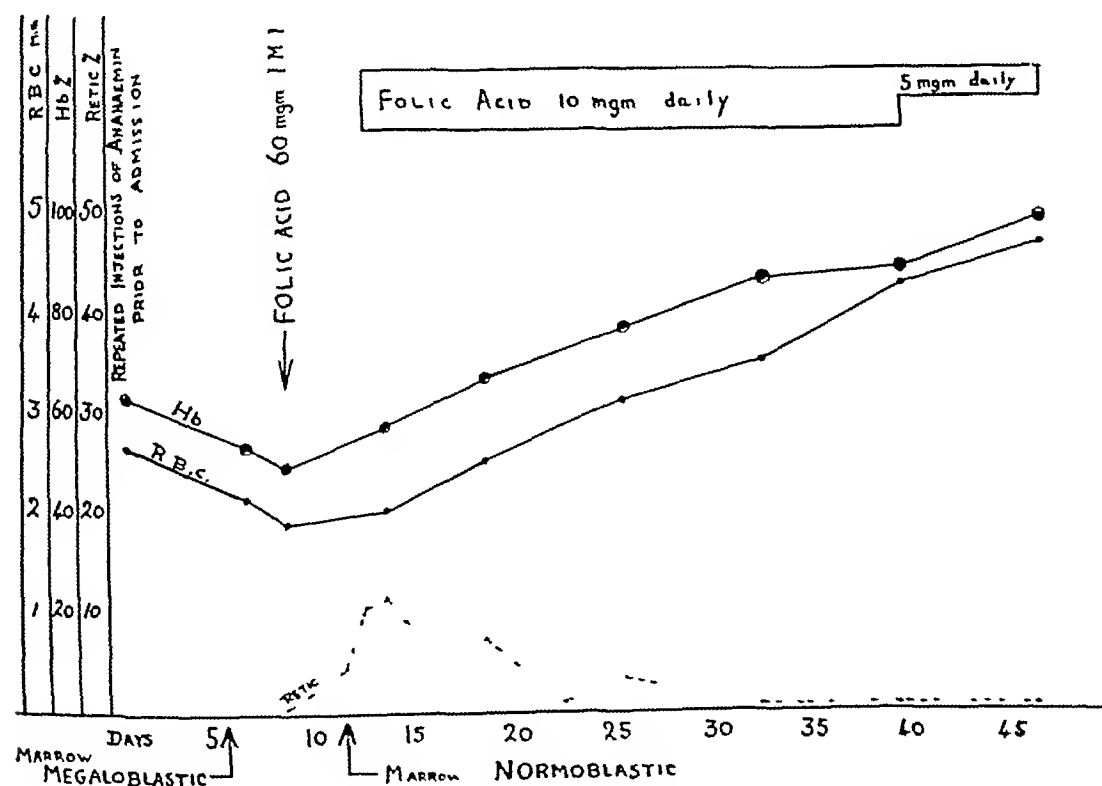


FIG 2 Male, aged 32, with refractory megaloblastic anemia associated with tropical sprue. Response to folic acid



with proteolysed liver and folic acid (figs 1, 2 and 3), and confine our observations essentially to the group which we have called "idiopathic refractory megaloblastic anemia"

A patient is placed in this group only if the cause of the megaloblastic anemia cannot be ascribed to direct dietary deficiency, pregnancy or the puerperium, malabsorption from the gastrointestinal tract or hepatic disease. It is obvious that the more thorough is the investigation and the more prolonged the period of observation the fewer will be the cases which will be classified as idiopathic. This point is well illustrated by the following 2 case histories

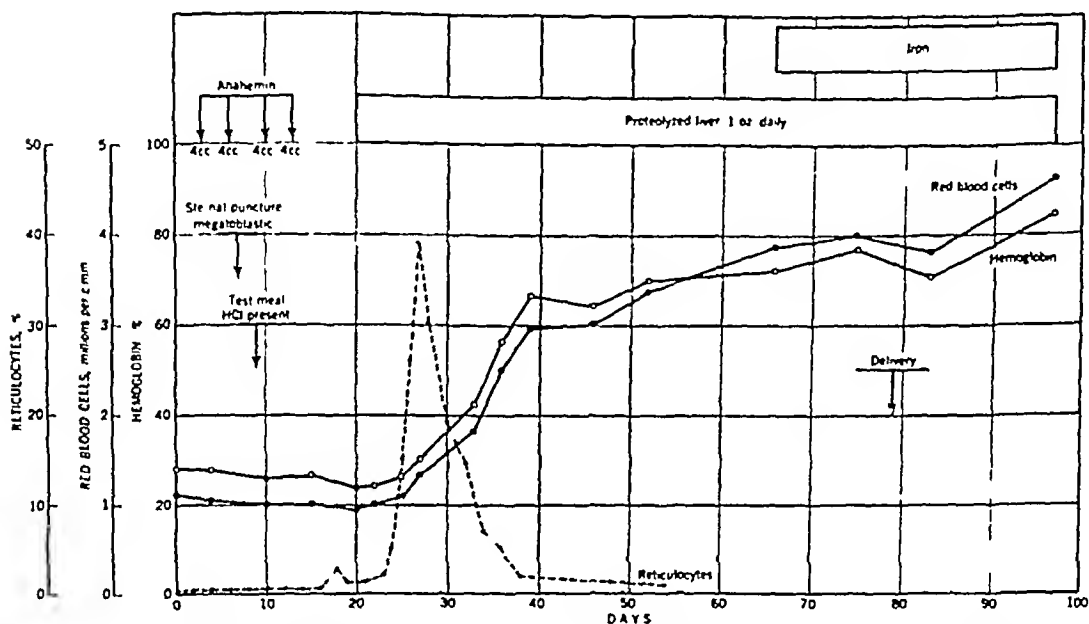


FIG 3 \* Refractory megaloblastic anemia associated with pregnancy Response to proteolysed liver

#### CASE HISTORY 1

*First admission* A man, age 61. He gave a two years history of weakness, breathlessness and anemia but there was no history of diarrhea, of paresthesia, of unsteadiness in walking, or of pain in the tongue. His diet had been satisfactory. A diagnosis of pernicious anemia was made in a neighboring hospital and the patient received 24 cc of a potent purified liver extract (Anahaemin) during a period of four weeks. The patient's condition continued to deteriorate so he was transferred to our clinic for further investigation and treatment.

When admitted the patient was very weak. His blood figures were as follows: Hb 28 per cent, RBC 950,000 per cu mm, WBC 5,200 per cu mm, PCV 13.0 per cent, MCV 136.8 cu microns, MCH C 30.0 per cent, reticulocytes 2.2 per cent, CI 1.5. The marrow was megaloblastic and a test meal showed that free hydrochloric acid was present. No other abnormality was found and at no time during the period of the first admission to our wards did the patient suffer from looseness of the bowels. He was treated with 20 mg of folic acid daily and this resulted in a reticulocyte peak of 18.2 per cent, a rapid gain in red cells and transformation of the marrow to the normoblastic state. Eventually the red cell count reached a level of 5 million. A diagnosis of idiopathic refractory megaloblastic anemia was made.

\* Figures 3 and 4 previously appeared in *Pernicious Anemia and Other Megaloblastic Anemias*, L. S. P. Davidson and L. J. Davis, in *Advances in Internal Medicine*, New York, Interscience Publishers, Inc., 1947, vol. II, pp. 481-547. Reproduced by permission of the publishers.

*Second admission* The patient returned to hospital a year later because his ankles were painful and swollen. He had been having repeated courses of folic acid and the red cell count was moderately satisfactory, being 4,110,000 per cu mm. Examination of the stools revealed that he was now passing two large motions daily and these were pale, greasy and bulky. A fat balance test was carried out and this showed that the patient was absorbing only 59 per cent of ingested fat (normal 90 to 95 per cent). This clearly indicated that the patient was suffering from a malabsorption syndrome and accordingly the diagnosis was revised to that of idiopathic steatorrhea.

#### CASE HISTORY 2

*First admission* A woman, age 37. Admitted to hospital in March, 1944, when she was five months pregnant. Her hemoglobin was then 56 per cent and red cells 2,050,000 per cu mm. The bone marrow was megaloblastic. A test meal showed the presence of free hydrochloric acid. A diagnosis of pernicious anemia of pregnancy was made. She failed entirely to respond to 4 cc of Anahaemin given intramuscularly, but responded to proteolysed liver, an increase of red cells of one million per cu mm occurring in twenty days. The patient was then discharged from hospital, but owing to difficulty in obtaining proteolysed liver she did not continue treatment.

*Second admission* She was readmitted in April, 1946, with a history of weakness and of intermittent diarrhea of a fatty type, a fat-balance test showed the percentage absorption to be 75 per cent. A test meal again showed the presence of free hydrochloric acid. She had never been abroad, and the dietetic history was normal. No antianemic treatment had been given for eighteen months before the commencement of folic acid therapy. At the start of folic acid therapy her blood findings were as follows: Hb 40 per cent, red cells 1,370,000 per cu mm, white cells 7,800 per cu mm, P C V 19.0 per cent, M C V 138.7 cu microns, M C H C 28.9 per cent, reticulocytes 3.5 per cent, C I 1.5.

The reticulocyte response and the rise in red cells over a therapeutic period of 14, 21 and 28 days reached the standards demanded by the U S P Board (see fig 1).

A diagnosis of idiopathic steatorrhea was made.

These case histories, together with others which we do not think it is necessary to elaborate, suggest that a failure in absorption from the alimentary tract may be a primary fault in some cases of idiopathic megaloblastic anemia. In a few of these patients the poor bodily build and lack of development of the skeleton suggested previous malabsorption from the gastrointestinal tract though no history of diarrhea could be obtained at any time from infancy up to the presenting illness. The absence of such a history may have little significance since we have clearly demonstrated in the sprue syndrome that fat absorption may remain grossly defective although diarrhea is absent and the patient's general health is good either as a result of folic acid therapy or from spontaneous remission (Davidson, Girdwood, and Innes, 1947). Other possible causes of alimentary dysfunction which are worthy of consideration are an abnormal intestinal flora or chemical or enzymic secretory defects which destroy the antianemic factor or fail to liberate it from its bound form in natural foods.

Some of the clinical and hematologic features of the group of cases labelled idiopathic refractory megaloblastic anemia are given in table 1. The patients were of both sexes and their ages ranged from 12 to 76 years. The chief complaint in all cases was weakness and breathlessness on effort, and physical examination usually revealed nothing of importance other than the signs and effects of severe anemia. Acute glossitis and objective signs of involvement of the central nervous system were absent. Chronic glossitis was frequently noted. The liver, spleen and lymphatic glands were not enlarged. The blood picture and the bone marrow were

identical with that seen in Addisonian pernicious anemia at corresponding levels of anemia, except in Cases 16, 17, 18 (see table 1). In those cases with histamine-fast achlorhydria the differential diagnosis from pernicious anemia was impossible.

TABLE 1—Cases of Idiopathic Refractory Megaloblastic Anemia

Case no	Sex	Age	Intr. HCl	Before treatment		Treatment given	Initial hematologic response to treatment
				Hb %	Red cells (mill./cu mm.)		
1	M	46	Absent	28	1 17	Intensive liver, iron, ascorbic acid, transfusion	Slow and delayed
2	M	20	Present	23	0 88		Slow and delayed
3	F	55	Absent	18	0 77		Slow and delayed
4	F	51	Absent	31	1 31		Slow and delayed
5	F	41	Present	22	0 86		Slow and delayed
6	F	34	Absent	18	0 75		Slow and delayed
7	F	45	Absent	32	1 91		Slow and delayed
8	F	34	Absent	28	0 89		Slow and delayed
9	F	51	Absent	27	1 34		Slow and delayed
10	M	14	Absent	44	1 80	Proteolysed liver	Prompt
11	M	12	Present	32	1 13	Proteolysed liver	Prompt
12	F	61	—	42	1 74	Proteolysed liver	Prompt
13	F	56	Absent	30	1 16	Proteolysed liver	Prompt
14							
1st admission	M	46	Present	36	1 24	Anahaemin	Slow and unsustained
2nd admission			Present	28	1 2	Proteolysed liver	Prompt
15	M	21	Present	46	1 77	Proteolysed liver	Prompt
16	F	76	Absent	22	0 97	Proteolysed liver	Moderate response
17	F	65	—	26	0 98	Proteolysed liver & anahaemin	Moderate response
18	M	58	Present	38	1 65	Proteolysed liver	Moderate response
19	F	72	—	32	1 13	Proteolysed liver	Moderately rapid
20	M	66	Present	45	1 82	Proteolysed liver	Prompt
21							
1st admission	M	60	Absent	50	1 93	Proteolysed liver	Prompt
2nd admission			Absent	58	1 99	Folic acid	Prompt
22	F	52	Absent	34	1 06	Folic acid	Prompt
23	F	70	Absent	44	1 93	Folic acid	Prompt
24	F	63	Present	52	1 78	Folic acid	Prompt
25	M	53	Absent	38	1 28	Folic acid	Prompt

Cases 16, 17, 18 These 3 cases had a dimorphic bone marrow. Although some typical megaloblasts were present the majority of the erythroblasts were normoblasts or cells intermediate in appearance between megaloblasts and normoblasts.

Case 19 Owing to vomiting this patient was initially unable to take adequate quantities of proteolysed liver.

until their failure to respond to parenteral injections of potent liver extract was discovered. In other cases the presence of free hydrochloric acid in the gastric juice was a point of exceptional diagnostic importance. Such cases would conform to the

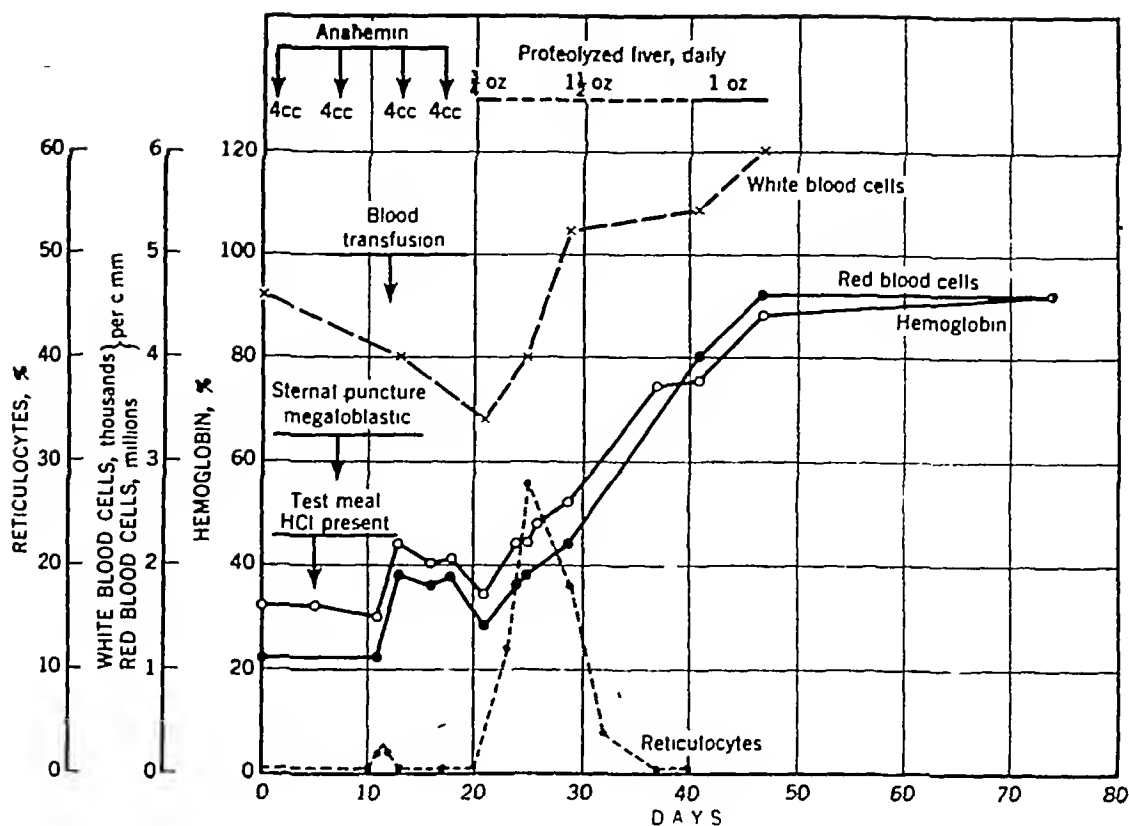


FIG 4 Idiopathic refractory megaloblastic anemia in a 12 year old girl Response to proteolysed liver

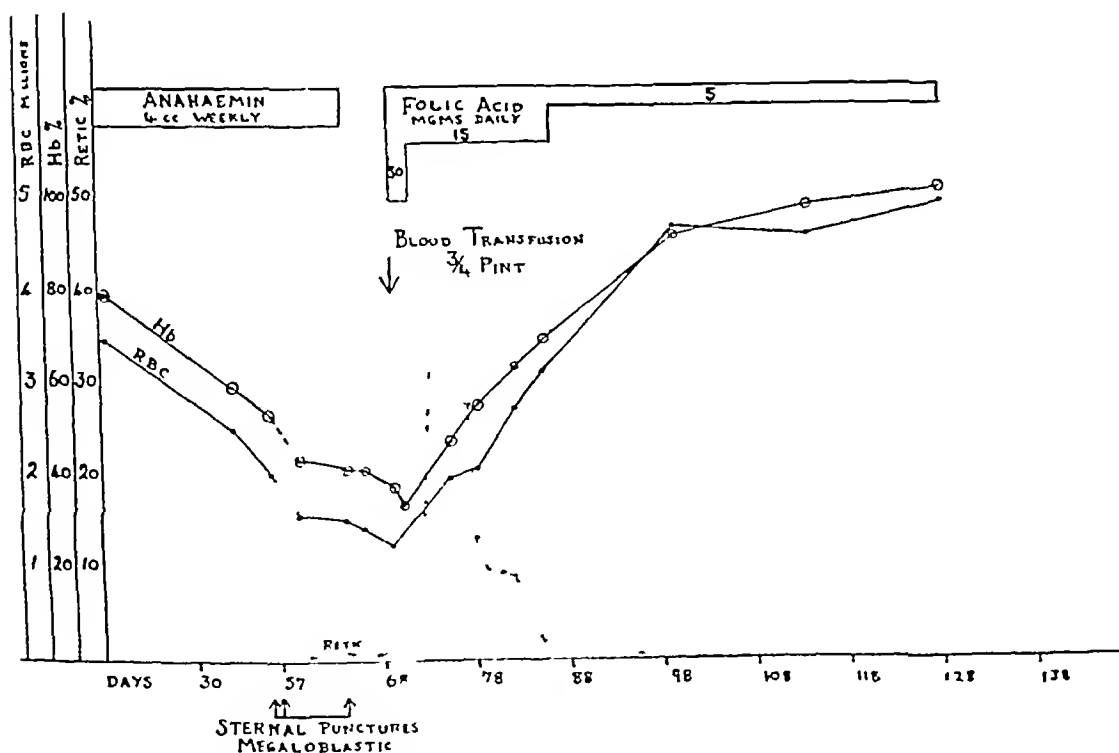


FIG 5 Idiopathic refractory megaloblastic anemia in a man aged 53 Response to folic acid

diagnosis of achrestic anemia as defined by Israels and Wilkinson (1936, 1940) When the disease occurs in patients below the age of 30 and is not associated with pregnancy, suspicion should be aroused that the case is not one of classic pernicious anemia Likewise, if the state of nutrition is unsatisfactory and the skeleton poorly developed a malabsorption syndrome should be suspected As has already been emphasized the patients were only placed in the idiopathic group when search for a cause for the anemia had failed to establish any satisfactory etiological explanation With regard to treatment it should be noted in table 1 that cases 1 to 9 were under our care prior to the advent of proteolysed liver and folic acid They had a prolonged and dangerous illness lasting weeks or months and many would have succumbed if life had not been supported by repeated blood transfusions while intensive treatment with parenteral liver extract, iron and vitamins was continued Cases 10 to 21 were treated with proteolysed liver, and cases 21 to 25 with folic acid Case 21 was treated successfully in his first relapse with proteolysed liver A second relapse, resulting from cessation of treatment, responded excellently to folic acid In contrast to cases 1 to 9 whose response to treatment was slow and prolonged, cases 10 to 25 responded to proteolysed liver or folic acid in a manner comparable to that obtained in pernicious anemia with parenteral liver extract therapy Representative examples of these cases are shown in figures 4 and 5

#### DISCUSSION

The parenteral injection of chemically purified liver extracts and the oral administration of liver, liver extracts, proteolysed liver and folic acid produce identical effects in transforming the megaloblastic bone marrow of Addisonian pernicious anemia into the normoblastic state as has been clearly demonstrated by studies of the bone marrow by ourselves and other workers It may therefore be assumed that some common factor is responsible for this effect Since daily doses of 1 mg. of synthetic folic acid will accomplish this result it appears likely that folic acid itself is the maturation factor, or plays some essential role in the final stage of the process of maturation Since purified liver extracts for parenteral therapy are practically devoid of folic acid it seems not unreasonable to suppose that they produce a transformation of the bone marrow through their ability to liberate free folic acid from its conjugated state Hence it may be postulated that in pernicious anemia the inability of the stomach to produce Castle's intrinsic factor leads to a failure in the production of an interaction product whose function is to liberate free folic acid There appears to be no failure in the absorption of conjugated folic acid since an immediate response is obtained to the injection of purified liver extract even when the patient has been partaking of an unsatisfactory diet for long periods

In contrast the refractory megaloblastic anemias may be considered to be due to a failure in the supply of conjugated folic acid since no response occurs to the injection of large doses of potent purified liver extract This failure of supply may result from direct nutritional deficiency as occurs particularly in tropical countries such as India (Lucy Wills, 1931) and the anemia may be partially or completely refractory to large doses of purified liver extract given parenterally Other cases can be explained on the basis of a malabsorption syndrome as is typically seen in tropical

sprue and idiopathic steatorrhea (see figs 1 and 2) As has already been noted some of these cases may not be recognized because a failure in absorption can occur in the absence of diarrhea In other cases the possibility exists that abnormalities in the intestinal flora, or chemical and secretory changes in the alimentary tract may destroy folic acid or make it unavailable to the body in some way as yet unknown

Lastly the liver plays an important role in the storage and possibly the final synthesis of the liberating factor formed from the interaction of Castle's intrinsic and extrinsic factors It may also be of importance in the storage and liberation of free folic acid Accordingly it is not surprising that in severe chronic disease of the liver a megaloblastic anemia is occasionally found In our experience, however, the macrocytic anemia of hepatic disease is accompanied much more frequently by a normoblastic marrow reaction Accordingly it may be concluded that a deficiency of conjugated folic acid can result from a variety of causes, some of which are known while others can merely be suspected The resulting megaloblastic anemia will be partially or completely refractory to parenteral liver extracts depending on the relative degree of deficiency of conjugated folic acid All types of megaloblastic anemia respond to the administration of free folic acid This is not surprising since by giving free folic acid the need for an interaction to take place between the liberating factor contained in purified liver extracts and conjugated folic acid contained in food is circumvented What is surprising is the dramatic therapeutic effect produced by the oral administration of folic acid in the malabsorption syndromes such as sprue We can only assume that the capacity to absorb different substances in this syndrome varies greatly Thus absorption of fat appears to be particularly poor while the absorption of free folic acid must be nearly perfect since a daily dose of 5 mg or less will produce the most dramatic clinical and hematologic improvement in sprue cases with a megaloblastic anemia

Lastly it is necessary to consider why proteolysed liver is usually as effective in the treatment of refractory megaloblastic anemia as is free folic acid

Proteolysed liver contains the liberating factor present in chemically purified liver extracts for parenteral use Experience has shown, however, that oral treatment with whole liver is very much less effective than parenteral treatment with liver extract derived from an equivalent amount of liver

The therapeutic effects of proteolysed liver in refractory megaloblastic anemia cannot therefore be ascribed to its content of this factor Proteolysed liver is a rich source of amino acids readily available for absorption because of the predigestion of liver protein with papain We have treated cases of pernicious anemia with a papain digest of beef protein without any response, and hence do not think it likely that the therapeutic activity of proteolysed liver depends on its content of amino acids It is possible, however, that liver contains some amino acid in high concentration which is necessary for the maintenance of normoblastic blood formation No evidence of this hypothesis is, however, available Supplementing the diet with individual amino acids such as methionine and choline has not been found effective in the treatment of the megaloblastic anemias Proteolysed liver is a rich source of many vitamins, especially of the vitamin B complex including folic acid The question therefore arises whether the therapeutic effects of proteolysed liver can be

ascribed to its content of folic acid. Before this question can be answered it would be necessary to undertake an assay of its folic acid content by the biologic methods at present in use. Opinion appears to differ widely among experts whom we have consulted in regard to the accuracy of such methods when used for the estimation of folic acid in foods and tissues. The only figures which we have available have been supplied to us by Dr. Riding of Evans Medical Supplies Ltd., who found an average figure of 0.8 mg. of folic acid per oz. of proteolysed liver. This quantity approaches the lowest amount of synthetic folic acid which we have found to be effective in the treatment of Addisonian pernicious anemia. As previously noted, of 5 cases given 1 mg. a day only 1 gave an optimal response, 2 showed no response and 2 showed a moderate response. In contrast the first 5 cases of pernicious anemia treated with proteolysed liver in doses of less than  $\frac{1}{2}$  oz. daily (folic acid content 0.4 mg.) all showed an optimal response. It may be safely concluded that the therapeutic effect of free folic acid must have been augmented by other hematinic principles contained in the preparation, e.g. the liberating factor and possibly other members of the vitamin B complex. The lowest dose of folic acid which we have used for the treatment of refractory megaloblastic anemia is 2.5 mg. daily and this dose was effective in the only case in which it was tried, hence we are unable to state what is the minimal effective therapeutic dose in this group of anemias. Even if it be assumed that it is in the region of 1 mg. daily the results achieved by 1 oz. of proteolysed liver daily in refractory megaloblastic anemia were superior to that produced by 1 mg. daily of synthetic folic acid in pernicious anemia. Accordingly we feel that the beneficial effects of proteolysed liver in refractory megaloblastic anemias cannot be explained solely on its content of folic acid, nor on its content of the liberating factor since large amounts of purified liver extract given parenterally are ineffective.

The superior efficiency of orally administered liver or proteolysed liver to liver extract given parenterally in refractory megaloblastic anemia can be explained on one or other of the following hypotheses: (1) That some interaction takes place in the gastrointestinal tract between the ingested liver preparation and gastrointestinal enzymes which leads to a potentiation of hemopoietic factors already present, or (2) that liver and proteolysed liver contain some essential hemopoietic principles, including folic acid and possibly other members of the B<sub>2</sub> complex, which are removed or destroyed in the chemical processes used in the manufacture of liver extracts for parenteral injection.

Additional support for this latter view is suggested by the following observations. We have many cases of tropical sprue and idiopathic steatorrhea who persistently have a moderate degree of macrocytic anemia which is entirely resistant to parenteral liver therapy. The bone marrow presents a picture which is mainly normoblastic but many erythroblasts have an appearance intermediate between a megaloblast and a normoblast. The failure of parenteral liver therapy and iron to restore the marrow picture to normal and the persistence of a macrocytic anemia clearly indicate a lack of some additional hematinic factor. The hope that this factor would be folic acid was not realized as can be clearly seen in the

protocols of our cases published in 1946 (Davidson, Girdwood, and Innes) Proteolysed liver also was found to be ineffective in some of these cases of refractory macrocytic anemia associated with the sprue syndrome In 2 or 3 cases, however, it caused a considerable increase in the blood level after parenteral liver therapy and folic acid by mouth had failed In such cases we concluded that proteolysed liver contained some additional hematinic principle whose composition was still unknown An investigation into the nature of this principle is proceeding Our therapeutic program in operation at the present time is based on the investigations detailed above Our practice is to treat all cases of megaloblastic anemia in the first instance with parenteral liver extracts Should the result be unsatisfactory we prescribe folic acid If this fails to restore the blood picture to normal we give 1 oz of proteolysed liver daily By this means we are able to restore the blood picture qualitatively and quantitatively to normal in the great majority of cases of megaloblastic anemia of all types

#### SUMMARY

1 Fifty-nine cases of megaloblastic anemia refractory partially or completely to potent liver extracts given parenterally have been investigated in Edinburgh during the past six years Thirty-four of these cases were associated with pregnancy, the puerperium or the sprue syndrome No explanation of the cause of the megaloblastic anemia was discovered in the remaining 25 cases

2 The etiology, clinical features and treatment of 25 cases of idiopathic refractory megaloblastic anemia are described Attention is directed to the excellent therapeutic effects produced by proteolysed liver or folic acid

3 The mechanisms involved in refractoriness to potent parenteral liver extracts are discussed

4 In certain cases of refractory megaloblastic anemia it is suggested that an unknown hematinic principle, in addition to the liberating factor in purified parenteral liver extract and folic acid, is required for the complete restoration of normoblastic blood formation

#### ACKNOWLEDGMENTS

My thanks are due to many members of my staff who have helped in these investigations, particularly to Professor L J Davis formerly lecturer in Medicine in the University of Edinburgh, and to Dr Girdwood Grateful acknowledgment must also be made to Doctor Riding, Medical Director of Evans Medical Supplies Ltd and his research chemists who were responsible for the preparation of proteolysed liver and the other fractions of liver mentioned above

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## FURTHER OBSERVATIONS ON THE SPECIFICITY OF THE FOLIC ACID MOLECULE

By TOM D SPIES, M D , GUILLERMO GARCIA LOPEZ, M D , ROBERT E STONE, M D ,\* FERNANDO MILANES, M D , ROBERT O BRANDENBURG, M D ,\* AND TOMAS ARAMBURU, M D

IT HAS been established that pteroylglutamic acid (folic acid) stimulates the development of white blood cells, red blood cells and platelets in a variety of animal species and in persons who have certain types of macrocytic anemia. Our studies of folic acid have been concerned chiefly with the striking hematologic response which follows its administration to persons with pernicious anemia, nutritional macrocytic anemia and tropical sprue in relapse. This response has been described in considerable detail,<sup>1</sup> and it has been pointed out that it is indistinguishable from that which follows the administration of refined liver extracts. Nevertheless, the potency of refined liver extracts is out of all proportion to the amount of folic acid they contain, and it is our working hypothesis that the hemopoietic factor in refined liver extracts differs chemically from folic acid per se. The study of the synthetic folic acid molecule offers great promise toward determining something of the nature of blood regeneration. Recently we showed<sup>2</sup> that patients who do not show a hematologic response to methyl folic acid will respond to the folic acid molecule. Since this study was reported, we have investigated the hemopoietic properties of six additional compounds, somewhat related to folic acid in their chemical structure, in persons with Addisonian pernicious anemia, nutritional macrocytic anemia and tropical sprue in relapse. This communication is concerned with these extended observations on the specificity of the folic acid molecule.

### MATERIALS AND METHODS

From a large group of patients, 11 patients with macrocytic anemia were selected for study. Four of these were classified as Addisonian pernicious anemia, 3 as nutritional macrocytic anemia, and 4 as tropical sprue patients. In all cases megaloblastic proliferation and defective maturation, a red blood cell count of less than 2.5 million and a color index of more than 1 were essential diagnostic criteria. For a differential diagnosis of the type of macrocytic anemia, additional criteria were the absence of free hydrochloric acid in the gastric contents even after histamine stimulation in pernicious anemia and the presence of free hydro-

Northwestern University Studies in Nutrition and Metabolism at the Hillman Hospital, Birmingham, Alabama, and at the Calixto Garcia Hospital, Havana, Cuba, in cooperation with the University of Havana. From the Department of Nutrition and Metabolism, Northwestern University.

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Valuable technical assistance was given by Miss Belle Culver, Miss Jane Davis and Mrs. Mary B. Koch.

\* Williams-Waterman Fellow in Nutrition.

chloric acid in nutritional macrocytic anemia and sprue. Usually the patients with nutritional macrocytic anemia had diarrhea characterized by loose, dark stools. The diarrhea present in the patients with sprue was characterized by large, liquid to semisolid, foul-smelling stools, varying in color from whitish yellow to yellowish green. A diagnosis of sprue was not made in the absence of steatorrhea. The glucose tolerance curve tended to be flat in both nutritional macrocytic anemia and sprue, and loss of weight, which usually had occurred in both, tended to be greater in sprue than it was in nutritional macrocytic anemia. A history of subsistence on an inadequate diet over a period of years was given by all of the patients with sprue and nutritional macrocytic anemia.

All the patients were admitted to the hospital where thorough medical and dietary histories were obtained and a complete physical examination was made. Rigid dietary control was instituted on admission and continued throughout the duration of the study. Meat, meat products, fish and poultry were excluded. Only 1 pint of milk, 1 hard cooked egg and 3 level teaspoons of butter were allowed daily. Raw vegetables were excluded and all other vegetables were served overcooked. All other foods were allowed in any amount desired.

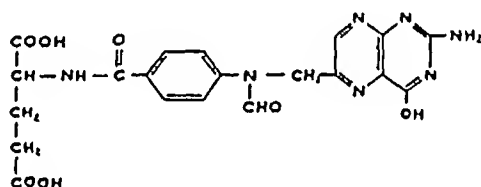


FIG. 1. FORMYL PTEROYL GLUTAMIC ACID (FORMYL FOLIC ACID)

For both the white cell and erythrocyte counts, certified Trenner pipets were used. The hemoglobin content of the blood was determined in grams by means of a Leitz or an Evelyn colorimeter. The reticulocytes were counted in wet preparations by the use of a modified brilliant cresyl blue solution of Dameshek. In all cases permanent fixed preparations of blood smears were made just prior to treatment, and once or twice a week thereafter cell volumes were determined on oxalated venous blood by means of Wintrobe hematocrit tubes. Prior to treatment, bone marrow was obtained and again at the peak of reticulocytosis, and still another specimen was obtained when the reticulocyte count returned to normal. Differential counts were made on preparations stained with both supravital and Wright-Giemsa stains.

After baseline determinations were completed, 20 mg of the Mg salt of formyl pteroyl glutamic acid (see fig. 1) was administered orally to 1 patient with pernicious anemia and to 1 patient with nutritional macrocytic anemia for ten days. Twenty mg of the Mg salt of formyl pteroyl glutamic acid (S. lactis factor) (see fig. 2) was administered orally to 1 patient with pernicious anemia for ten days. Twenty mg of N-(4-(4-quinazoline) amino) benzoyl)-glutamic acid (see fig. 3) was given orally to 1 patient with pernicious anemia for ten days. Then the dose was increased to 50 mg daily for five days. Twenty mg of pteroyl aspartic acid (see fig. 4) was given orally to 1 patient with tropical sprue for ten days, and 40

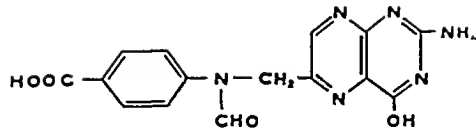


FIG 2 FORMYL PTERIOIC ACID (S LACTIS FACTOR)

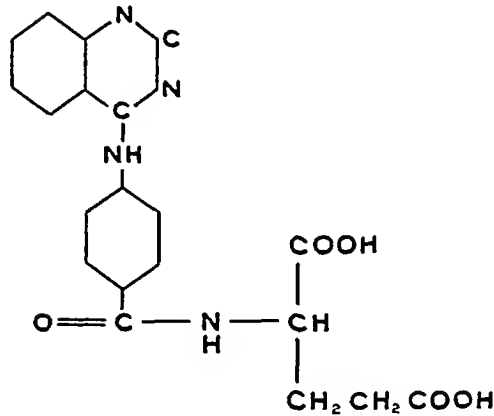


FIG 3 N-(4-(4-QUINAZOLINE)AMINO)BENZOYL)-GLUTAMIC ACID

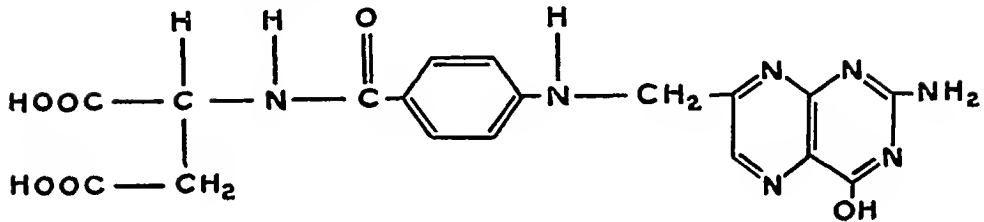


FIG 4 PTEROYL ASPARTIC ACID

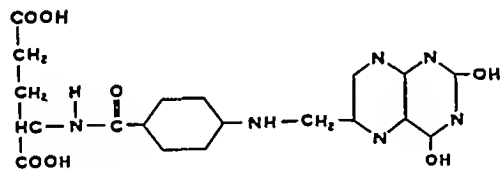


FIG 5 OXYPTEROYL GLUTAMIC ACID (OXYFOLIC ACID)

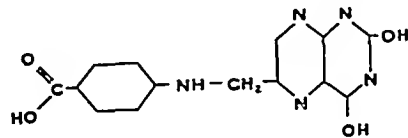


FIG 6 OXYPTEROIC ACID

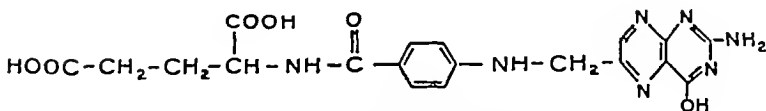


FIG 7 LIVER L CASEI FACTOR

N-[4-[(2-amino-4-hydroxy-6-pteridyl)methyl]amino]benzoyl] glutamic acid

mg was given daily for an additional ten days. Another patient with tropical sprue was given 20 mg orally for ten days. Twenty mg of oxyfolic acid (see fig 5) was

given daily by mouth to one patient with nutritional macrocytic anemia and to 1 patient with pernicious anemia for ten days. Twenty mg of oxypteroic acid\* (see fig 6) was given daily by mouth to 2 patients with tropical sprue. If, within ten days, reticulocytosis had not occurred, 10 mg of folic acid (see fig 7) was administered until the blood values reached satisfactory levels.

## RESULTS

*Response to Mg Salt of Formyl Pteroyl Glutamic Acid* Following the administration of this material to the patient with pernicious anemia, the reticulocytes began to rise on the seventh day and reached a peak of 9.6 per cent eleven days after its administration was initiated. This was followed by a slight increase in red blood

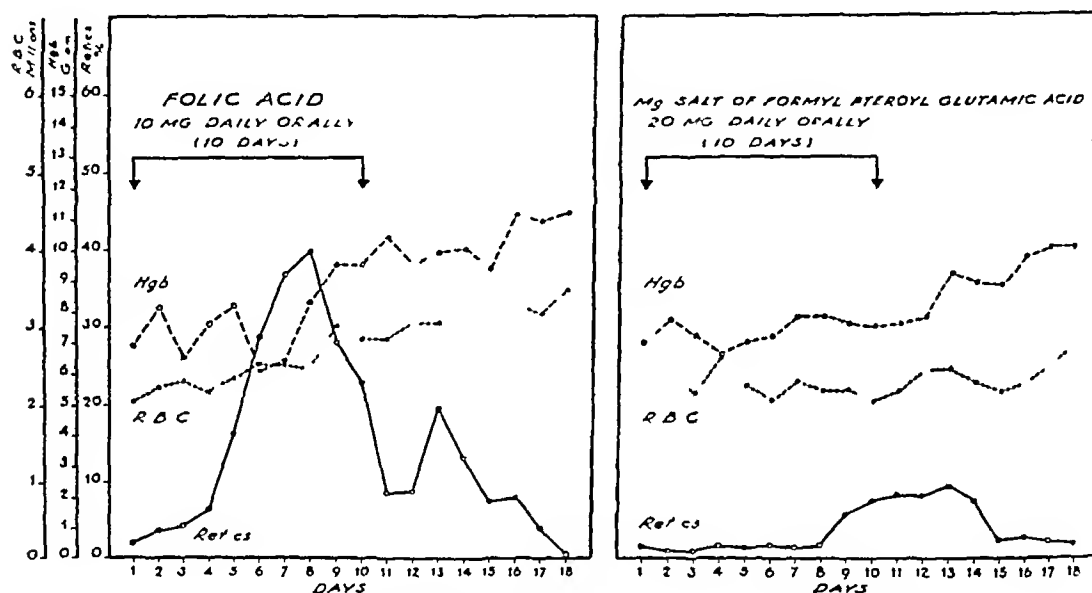


FIG 8 Comparative response to folic acid and Mg salt of formyl pteroyl glutamic acid in a patient with addisonian pernicious anemia

cells, hemoglobin, white blood cells and platelets. The response, however, was poor compared to his response to the administration of folic acid per se during another comparable relapse of the disease when his reticulocytes began to rise on the fourth day of therapy and reached a peak of 39.8 per cent on the eighth day (see fig 8). By the end of ten days there was an increase in white blood cells and platelets. The response of the patient with nutritional macrocytic anemia to Mg salt of formyl pteroyl glutamic acid was slightly greater, but it was not of the magnitude which followed folic acid per se. Following the administration of Mg salt of formyl pteroyl glutamic acid, the reticulocytes began to rise on the fourth day and reached a peak of 25.4 per cent on the tenth day, whereas on folic acid per se,

\* The Mg salt of formyl pteroyl glutamic acid and the Mg salt of formyl pteroyl glutamic acid were furnished by Dr. Y. Subbarow of Lederle Laboratories, Inc. The N-(4-(4-quinazoline) amino) benzoyl)-glutamic acid, the pteroyl aspartic acid, the oxypteroic acid and the oxypteroic acid were furnished by Dr. Gustav Martin of The National Drug Company. Only small quantities of these compounds were available so that in no instance did we have an opportunity to test the effect of massive doses.

which was administered during another, but comparable, relapse of the disease, the reticulocytes began to rise on the third day and reached a peak of 60.4 per cent on the eighth day (see fig 9). There was a slight rise in the red blood cells, hemoglobin, white blood cells and platelets following the administration of the Mg salt of formyl pteroyl glutamic acid but they did not reach satisfactory levels until folic acid therapy was initiated.

**Response to Mg Salt of Formyl Pteroyl Glutamic Acid (*S. lactis* Factor)** The administration of this material in the dosage given did not produce blood regeneration in a patient with pernicious anemia, whereas an excellent response followed the administration of folic acid per se.

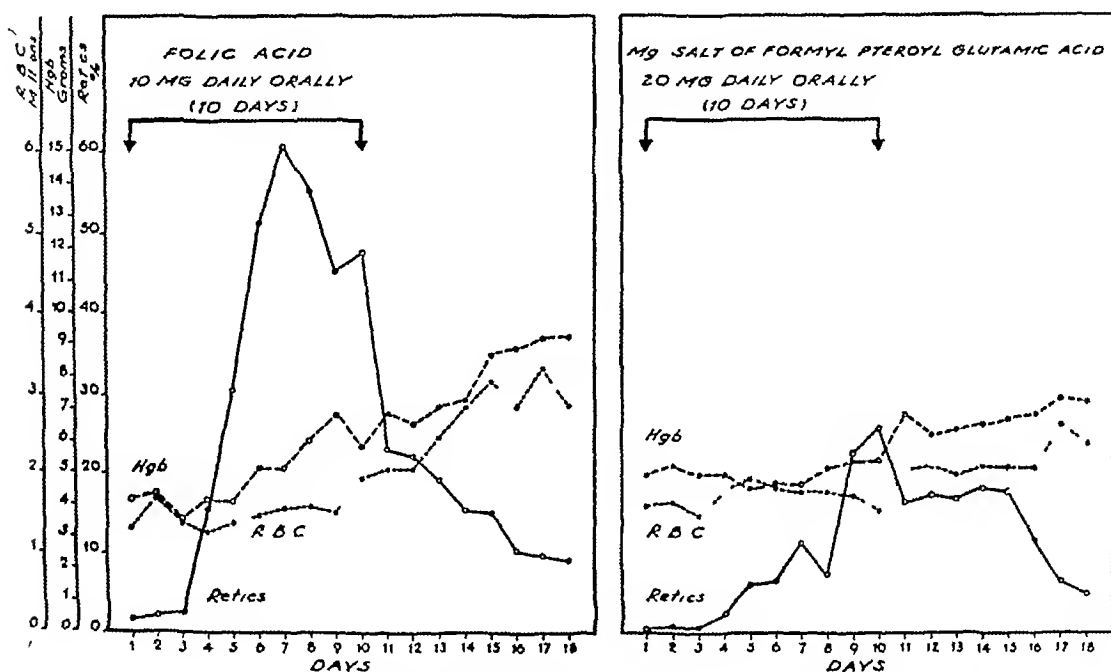


Fig 9 Comparative response to folic acid and Mg salt of formyl pteroyl glutamic acid in a patient with nutritional macrocytic anemia

**Response to N-(4-(4-Quinazoline) amino) benzoyl)-Glutamic Acid** Neither the patient with pernicious anemia nor the one with nutritional macrocytic anemia had any hematologic response to this material at the dosage level used. On subsequent therapy with folic acid an excellent response was observed.

**Response to Pteroyl Aspartic Acid** Blood regeneration did not follow the administration of this material at the dosage level given in either of the 2 cases of tropical sprue. Both these patients later showed a good response to folic acid.

**Response to Oxyfolic Acid** The administration of this material produced no blood regeneration in the patient with pernicious anemia or in the one with nutritional macrocytic anemia at the dosage level administered. Subsequent treatment with folic acid was followed by an excellent response.

**Response to Oxypteroyl Acid** At the dosage level used, blood regeneration did not follow the administration of this material in two cases of tropical sprue, whereas folic acid therapy which was given later produced an excellent response.

## SUMMARY AND CONCLUSIONS

Methyl folic acid,<sup>1</sup> N-(4-(4-quinazolinc) amino) benzoyl)-glutamic acid, the Mg salt of formyl pteroyl glutamic acid, the Mg salt of formyl pteronic acid, pteroyl aspartic acid, oxyfolic acid and oxypteronic acid have been studied as to their effect on blood regeneration in selected cases of Addisonian pernicious anemia, nutritional macrocytic anemia and tropical sprue. In the amounts administered, only the Mg salt of formyl pteroyl glutamic acid was effective in producing reticulocytosis and an increase in red blood cells, hemoglobin, white blood cells and platelets, and it was not as effective per unit of weight as was folic acid per se. Presumably this compound is slowly changed into folic acid in the body. It is of special interest that the Mg salt of formyl pteronic acid (*Streptococcus lactis factor*) was negative in producing hemopoiesis. These observations show the very great specificity of the folic acid molecule.

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# CASE REPORT

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## A CASE OF PERNICIOUS ANEMIA REQUIRING ENORMOUS AMOUNTS OF LIVER, ESPECIALLY BY MOUTH, OVER TWENTY YEARS

By ROGER I LEE, M D

I STILL believe that there is a place for the occasional case report in medical literature. A disease like pernicious anemia, with its protean manifestations and wide variations of course, presents difficulties in the way of statistical presentation. I have used the present case for clinical demonstration a number of times. If we both live, her case may be continued.

At first I used to demonstrate this patient, who needed a large amount of liver over a good many years for the treatment of pernicious anemia, as a contrast to another patient who needed liver only once a month or so. There was, I think, no possible doubt of the diagnosis in the 2 cases, but the contrasting case unfortunately died of an intercurrent disease.

### REPORT OF CASE

The case which I report is that of a woman who was seen first in March 1925. She was a school teacher, then 39 years of age. She had symptoms of fatigue and weakness, with irregular fever beginning in 1923. When she was first seen, the platelets were very much increased and a positive diagnosis of pernicious anemia was not made until August 1925. The feature of her early course was a continued fever. She was put on a diet of raw liver, but escaped observation by going to another part of the country where liver was discontinued. In 1928 she developed trouble with her legs. In 1929, she presented a fairly typical ataxic paraplegia. She was given one-half pound of raw liver a day with a great deal of improvement in her ability to walk, although she still presented the clinical picture of ataxic paraplegia. Since this episode and to the present time, she has required a very large amount of liver. She has had liver in every available form. If she did not have some liver by mouth and tried to depend entirely upon injections of liver, slowly she would develop headache, backache, and some slight difficulty with her bladder. When she increased the liver by mouth, these symptoms would slowly subside. All the time she was taking injections of liver extract, usually once a week and sometimes twice. During the war years when liver was difficult to obtain, this patient had a particularly hard time in securing the liver to be taken by mouth. She did very well on half a pound of beef liver (she could not take pig's liver) six days a week. That would represent a yearly intake of 150 pounds of liver, by mouth. This woman weighed only 100 pounds on the average, sometimes less and sometimes more. Consequently, she ate her weight in liver in a year and this was in addition to the injections of liver. In the twenty odd years which she has been under treatment, she has taken by mouth certainly over a ton of raw liver. In addition, we have given her iron, all forms of liver substitutes by mouth and every form of liver extract. None has had the same effect as the raw liver by mouth. We have given her vitamins, all without any appreciable effect. We have also given her folic acid and this too in addition to the injections of liver and what liver she can take by mouth. We have no final opinion on the effect of folic acid yet, but it does seem to be beneficial. During all this time, her hemoglobin has been running from 84 to 94 per cent and her red cell count four and a half to five million.



A very curious feature has been that she would develop headache, back pain and some increased disturbance of the bladder and in walking long before she showed any evidence of disturbance in her blood. Gradually, the blood would show evidences of a slight deterioration, but this was never very marked. The improvement of symptoms would anticipate by a month or two the improvement of her blood. These down dips in her symptoms were usually initiated by an intercurrent infection or some complication which made it difficult for her to get the additional liver to take by mouth.

Anyone who has studied pernicious anemia appreciates the wide variation in the spontaneous course of the disease. In this case, it is to be noted that fever was an early symptom and that this was by no means slight. I regard that as an indication of the intensity of the condition. The contrasting case, which I used to show with this patient, at no time had fever. Her condition seemed to be of a gentler kind. The patient in this contrasting case, in spite of the small amount of liver that she took, due to the fact that she felt well, never developed the lesions of pernicious anemia in the central nervous system. I think it has long been recognized that the more intense cases of pernicious anemia are more likely to have lesions of the central nervous system, although it is well known that all long-standing cases of pernicious anemia have at autopsy actual lesions in the central nervous system. Our patient developed symptoms of the central nervous system early. At one time, there was a great deal of discussion as to the efficacy of liver therapy in lesions of the central nervous system. This case would seem to indicate that liver therapy does not cause complete regression of the lesions of the central nervous system, but it keeps them under control.

At no time during these twenty years, has this patient had an enlarged liver, enlarged spleen, hypertension or particularly abnormal blood chemistry, although the non-protein-nitrogen was apt to be in the high 30's and the uric acid was apt to be around four.

This case indicates several things to me:

1. The variation in the intensity of the disease of pernicious anemia.
2. That involvement of the central nervous system is apt to indicate a disease that may be difficult to treat.
3. That some cases of pernicious anemia require relatively enormous amounts of liver therapy and that successful therapy may demand even in these days a combination of oral as well as intramuscular liver.
4. Finally, this case illustrates the fact that some cases of pernicious anemia present real problems in treatment and cannot be treated by any rule of thumb method.

# BLOOD

## *The Journal of Hematology*

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### THE SCHOENLEIN-HENOCH SYNDROME OF VASCULAR PURPURA\*

By ELI DAVIS, M D

#### DEFINITION

THE Schoenlein-Henoch syndrome is a condition in which nontraumatic hemorrhage, with or without edema, occurs in the skin, or subcutaneous tissue, or joints, or viscera, or in any combination of them, and in which the blood platelets are normally abundant

The name Schoenlein-Henoch purpura is well established, yet the eponym is ill chosen (as so many eponyms seem to be) Willan<sup>14</sup> described one type of the syndrome in a London publication in 1808, while a description of Schoenlein's disease was recorded not by Schoenlein himself but by one of his pupils in 1837<sup>11</sup> According to this description, the patients were often rheumatic sufferers, and they presented purpura, pains, and swellings, particularly in the knee and knuckle, but occasionally in the hand and shoulder joint Henoch's work appeared in 1868<sup>7</sup> and he referred to 7 cases in a paper in 1874<sup>8</sup> Apert<sup>1</sup> (1897) gave an excellent clinical description under the name of exanthematic purpura Osler,<sup>9</sup> in 1914, in his paper, "The Visceral Lesions of Purpura and Allied Conditions," did much to clarify ideas on the condition, but his view that the anaphylactic key would unlock the mystery of these cases found too ready an acceptance

Numerous case reports have appeared, but most observers have described but few cases each and only a hazy idea of the natural history has emerged The description which follows is based on 44 personally observed cases seen in eight years, against a background of 1200 cases of purpura (mostly benign) An account of the first 500 of these cases has been given by Davis<sup>3</sup> (1943)

#### SEX, AGE, DURATION

The condition was about three times as common in women as in men, but, in children, boys and girls were about equally affected Patients aged 4 to 71 were affected, but there were peaks of incidence of first attacks from 4 to 10 years old, and from 16 to 40 Half of the patients had had more than one attack, and half were seen in their first attack It is probable that about one third had had but one attack A few had had numerous attacks Each attack usually lasted for a few weeks Attacks had recurred over periods varying from a few months up to 50 years,

\*From the Rothschild Hadassah University Hospital, Jerusalem Most of the cases on which this paper is based were seen in the service of the London County Council, but typical cases seen in Jerusalem are included

but in most patients they ceased within five years from the onset. Of the 44 cases, 5 patients were each members of different families with hereditary familial purpura simplex. No familial instances were encountered.

### ETIOLOGICAL FACTORS

In 10 patients attacks were preceded by acute tonsillitis. In one patient, each of her three attacks was heralded by an acute tonsillitis. One patient suffered from



FIG. 1. Girl, aged 8 (a) Edema of eyelids and face (b) Petechiae of buttocks. Melena, hematuria, joint swellings

pulmonary tuberculosis and had two attacks. In another patient true acute cholecystitis preceded the attack. There was a history of acute rheumatic fever earlier in life in 4 patients, but in at least 2 of these patients the so-called acute rheumatism was almost certainly an earlier attack of Willan-Schoenlein-Henoch disease. Only 6 of the patients were affected with fibrositis. In 8 patients an antistreptolysin titer was done at the height of the disease, in 6 the titer was normal, and in 2 raised to 800 and 1250 units. (Several of the patients with acute tonsillitis had normal titers.)

## TEMPERATURE

Only 11 patients were febrile during attacks. Three were febrile for one month, another had low grade fever for one year, a fifth had bouts of low grade fever lasting about a week every few months for over five years, and a sixth had persistent fever of up to 100-101° for months at a time for the eight years she was under observation. Blood cultures were negative in all.



FIG 2 Girl, aged 7 Petechiae back of neck and shoulder, blood in cerebrospinal fluid, melenæ, joint swellings

## PURPURA, RASHES, EDEMA

Of the 44 cases, 38 showed spontaneous ecchymoses or petechiae or both. Most showed skin purpura only during attacks but a few had ecchymoses between attacks. The ecchymoses, which occurred mostly on the limbs, varied from  $\frac{1}{2}$ -4 inches in diameter (fig 4) and were painless. Petechiae were seen, particularly over the skin of the buttocks and scapulae (figs 1 and 2), but the number of petechiae varied enormously, and the widespread rash with dense petechiae (fig 5) was rare. Petechiae were also seen in the mucous membrane inside the mouth and occasionally on capillary microscopy of the nail bed.<sup>4</sup> In two patients the purpura

was in the form of a purpura nodosum, the erythema of erythema nodosum being replaced by purpura (fig 3) Melena was seen in 5 patients Epistaxis, hematemesis, and menorrhagia each occurred in 4 patients, hematuria in 3, mouth bleeding in one, blood was found in the cerebrospinal fluid of one patient with meningeal irritation<sup>5</sup>

Urticaria accompanied the attacks in 3 patients and 4 others had a papular or papulovesicular rash Localized edema occurred in the following order of fre-



FIG 3 Woman, aged 22 Ecchymoses of legs, purpura nodosum, hematuria, febrile one year, joint swellings

quency backs of hands, around the eyes, face (fig 1a), neck, lips, legs, arms, and penis

#### JOINTS, VISCERA

Joints particularly involved were those of the fingers, wrists, knees, elbows, and ankles Any one or any combination of these joints could be affected Joints were painful and nearly always swollen In some patients the edema was peri-articular and para-articular rather than in the joint itself Between attacks all affected joints recovered full function and did not ankylose, but in a few patients pain persisted Radiological changes did not develop

Gastrointestinal pain or hemorrhage was pronounced in 12 patients, while 3

patients had hematuria, a fourth casts and albuminuria without hematuria, and one patient gallbladder disease. Menorrhagia was present in 4 patients and uterine pains in a fifth. The spleen was not palpable.

#### GENERAL

The time relationship of the features of an attack was very variable. Petechiae, edema, joint pains, and visceral features often appeared within hours of one another, but skin petechiae at times preceded or followed other signs by an interval



FIG. 4. Woman, aged 40. Ecchymoses, febrile eight years, joint swellings.

of several days. Thus a puzzling visceral pain or joint swelling could be diagnosed only when a purpuric rash developed days later, or the nature of a purpuric rash might remain obscure until swollen fingers, orbital edema, and melena occur. In some cases, skin manifestations were absent entirely. By way of contrast, angioneurotic edema might be the sole feature of an attack. A classic illustrative case from this series has been described by Green.<sup>5</sup>

#### BLOOD FINDINGS

Blood counts, platelet counts, bleeding and coagulation times, clot retraction, and prothrombin time were normal but occasionally a polymorphonuclear leuco-

cytosis was seen. Blood sedimentation rate was usually normal, but occasionally high, likewise the antistreptolysin titer. When these were high there was also leucocytosis. The capillary resistance test was positive in only some 25 per cent of cases.

### PROGNOSIS

The outcome for a particular attack and the ultimate outcome were good. Recovery was invariable, good health was enjoyed between attacks, and in most

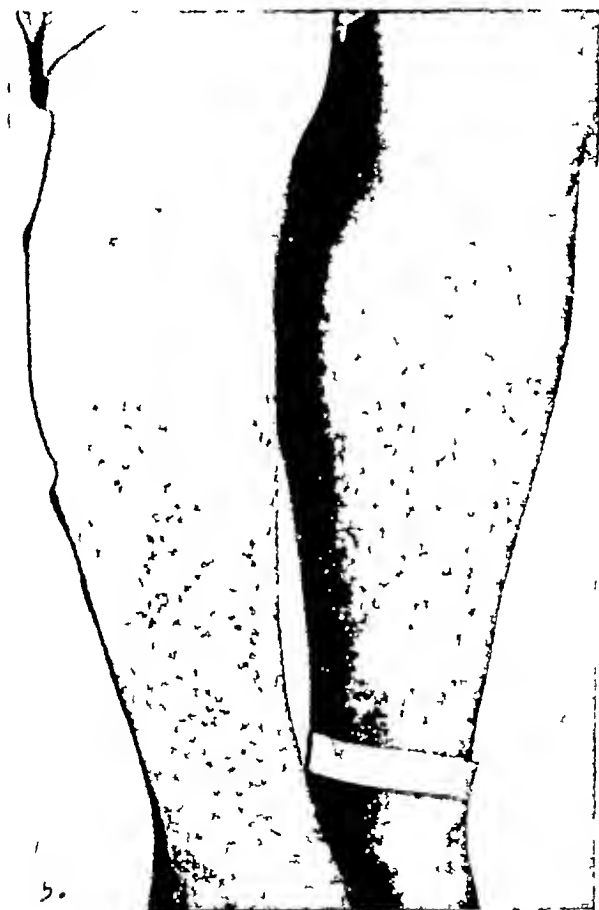


FIG. 5. Man, aged 30. Massive petechiae legs, hematuria, joint swellings.

patients attacks rarely recurred after 5 years. The 4 patients with renal involvement had no residual lesions and made full recoveries despite the gloomy prognosis so often given to Schoenlein-Henoch purpura with renal complications. The child with the subarachnoid hemorrhage recovered completely.<sup>5</sup> But some patients required treatment for hematuria or hematemesis or melena, which they naturally found alarming. Persistent fever in several patients was worthy of note, particularly in the case presenting pyrexia lasting for eight years. This experience demonstrates that Schoenlein-Henoch purpura must be added to the causes of persistent low grade fever.

## DIFFERENTIAL DIAGNOSIS

It is clear that abundant opportunities for confusion with other diseases exist. Among the labels attached to cases I encountered were acute rheumatic fever, septicemia, subacute infective endocarditis, rheumatoid arthritis, infective arthritis, nephritis, bleeding peptic ulcer, and trichinosis. The differential diagnosis from palindromic rheumatism<sup>6</sup> must also be considered. The cases of angioneural arthrosis described by Solis-Cohen<sup>13</sup> (1914) may be identical with Schoenlein-Henoch purpura.

## TREATMENT

Treatment was essentially symptomatic. Vitamin C was of no value, the preparations of vitamin P used were not beneficial, and vitamin K was not indicated. The patient with persistent attacks of fever for one year recovered after tonsillectomy. The patient with the eight years' fever was not helped by protein shock, auto-hemotherapy, blood transfusions, or sulphonamides. After the exhibition of penicillin an attack was cut short and she enjoyed three months of exceptional well-being, fever-free. But a second course of penicillin given for the relapse was ineffective. Spontaneous recovery had occurred after protracted attacks in the pre-penicillin era.

## DISCUSSION

Most authors regard purpura simplex and Schoenlein-Henoch purpura as different facets of the same condition. By purpura simplex, I understand a condition in which ecchymoses with or without petechiae occur from time to time in the skin without any known trauma and without ascertainable cause. Now there is no doubt that one group of Schoenlein-Henoch cases is closely linked with purpura simplex—witness the occurrence of a case of Schoenlein-Henoch purpura in five different families of hereditary purpura simplex, while some cases of purpura simplex become almost indistinguishable from Schoenlein-Henoch purpura. The blood findings in both conditions are identical. There are distinct differences, however. Purpura simplex is overwhelmingly more frequent in women, is very often familial and is not common in children, but Schoenlein-Henoch purpura is not uncommon in males, is not familial and is quite common in children. One third of the patients with Schoenlein-Henoch purpura have only one attack, but single attacks of purpura simplex are much rarer. The fibrositic diathesis is much commoner in purpura simplex. My conclusion is that Schoenlein-Henoch purpura is a syndrome and not a disease and is a nonspecific reaction to different factors. The causes of purpura simplex and Schoenlein-Henoch purpura overlap but are not identical. One great cause of both conditions is recent infection by hemolytic streptococci. (It is possible that in some instances Schoenlein-Henoch purpura represents aborted or modified rheumatic fever, sparing the heart. Osler<sup>9</sup> stated that rheumatic poison is believed to be responsible for a large group of these cases.) In most of my cases the cause was not found. In none of my cases of Schoenlein-Henoch purpura were food or drugs or known allergens found to be the cause,



but I would agree that allergic factors cause this condition occasionally. It is to be noted that no support for the anaphylactoid etiology was given by Battle and Bell<sup>2</sup> (1936), Poncher<sup>10</sup> (1935), and Siedlmayer<sup>12</sup> (1939). Osler<sup>9</sup> was optimistic in his belief in the anaphylactic key, but he was right to insist that urticaria, angioneurotic edema, and vasomotor instability were all aspects of the same condition.

### SUMMARY

The Schoenlein-Henoch syndrome is described on the basis of 44 personally observed cases. It is defined as a condition in which nontraumatic hemorrhage with or without edema may occur in the skin, or subcutaneous tissue, or joints, or viscera, or in any combination of them, and in which the blood platelets are normally abundant. The causes of the syndrome are varied, but streptococcal infection is important, and "anaphylactoid" causes rare. The prognosis is good, and the 4 cases with renal involvement also did well. Schoenlein-Henoch purpura and purpura simplex overlap, but in contrast with the latter, Schoenlein-Henoch purpura is not familial, and is common in males and children.

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# HYPERPROTHROMBINEMIA INDUCED BY VITAMIN K IN HUMAN SUBJECTS WITH NORMAL LIVER FUNCTION

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**H**YPERPROTHROMBINEMIA, as determined by reduction of prothrombin time below the range of normalcy, has been observed in man in cases of intravascular thrombosis and in animals after the administration of vitamin K.<sup>1-9</sup> The present study was undertaken to learn whether it is possible to induce hyperprothrombinemia in human subjects with normal liver function by the administration of large doses of menadione derivatives.

## MATERIAL

Thirty-eight persons were studied. Students, house officers, and nurses made up 21 of the group. The remaining 17 were patients in whom liver function was normal. These were almost all cases of essential hypertension uncomplicated by circulatory or renal failure. All showed normal prothrombin time on repeated examinations. One case had amyloidosis in which a previous survey had yielded changes in prothrombin time pertinent to the present investigation. The data recorded below were obtained more recently.

## METHOD OF PROTHROMBIN ASSAY

The procedure used was that described in earlier communications of the series.<sup>1, 8</sup> It includes estimation of the prothrombin time of whole and diluted (12.5 per cent) plasma. For reasons given below, in 11 of the cases the prothrombin time of 8 per cent plasma was estimated. Venous blood was used exclusively. Each sample was freshly drawn and the plasma maintained in the water bath at 37° C for approximately fifteen minutes before the prothrombin time was estimated. All estimations were made in duplicate. The thromboplastin was prepared by vacuum desiccation of fresh rabbit lung. The end point was determined by lifting the clot, the instant it formed, away from the otherwise clear liquid by means of a tiny loop made of no. 22 nichrome wire.

## PROCEDURE

The existing value of prothrombin time was established. Following this, synthetic vitamin K in the form of Hykinone\* (Abbott) or Synkayvite† (Hoffmann-LaRoche) was administered by intravenous injection, and the prothrombin time was estimated daily for at least three succeeding days. In the cases in which no changes were detected, the experiment was continued for at least five days. Changes occurred in the diluted plasma prothrombin estimations only, and consequently the data presented below are confined to these figures.

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\* Hykinone = 2-methyl-1,4 naphthohydroquinone sodium bisulfite.

† Synkayvite = 2-methyl-1,4 naphthohydroquinone diphosphoric acid ester tetrasodium salt.

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## RESULTS

The data of the two groups are given below. In the first are 15 representative cases in which the 12.5 per cent plasma was studied and in the second are 7 subjects in which the 8 per cent plasma showed significant reduction of the prothrombin time after vitamin K medication.

The prothrombin clotting time was first estimated before the administration of the vitamin K preparation. When more than one figure was obtained, the arithmetic average of these figures was used. All were within the normal range. Prothrombin time estimations were made following the injection of the water soluble vitamin K preparation. The lowest prothrombin time after the injections was used to calculate the effect.

TABLE 1

	Prothrombin Time Mean	S D	S.E. <sub>m</sub>
	sec		
I Before vitamin K	40.13	±2.92	±0.75
II Lowest after vitamin K	36.13	±2.68	±0.69
III 48 hours after last dose	39.25	±2.96	±0.76

S D = standard deviation

S E<sub>m</sub> = standard error of the mean

TABLE 2

Difference	S E <sub>Diff</sub>	C R
I and II 4.00	±1.02	3.9
I and III 0.88	±1.07	0.8
II and III 3.12	±1.02	3.2

Difference = difference between means of each group

S E<sub>Diff</sub> = standard error of the difference

C R = critical ratio

The mean, standard deviation and the standard error of the mean were calculated for each group of figures. The difference between the mean of each group and the reliability of these differences was also calculated.

## INTERPRETATION OF STATISTICS

The difference between the means of group I and II and of groups II and III is statistically reliable. From this we may conclude that there is less than one chance in a thousand that the difference is due to chance.

The difference between the means of groups I and III is not statistically reliable.

It is concluded that the effect of the administration of synthetic vitamin K was to reduce the prothrombin time significantly to the level of hyperprothrombinemia.

One advantage in using diluted plasma is to amplify the changes which occur

TABLE 3

Case No	Day	Prothrombin Time of Diluted (12.5%) Plasma	Intravenous Medication
1		<i>sec</i>	
	1	45	Hykinone* 20 mg
	2	41	Hykinone 20 mg
	3 4	37 44	
2	1	39.4	Hykinone 20 mg
	2	39.0	Hykinone 20 mg
	3	39.5	
	4	41.3	
3	1	39.0	Hykinone 20 mg
	2	29.0	Hykinone 20 mg
	3	35.0	
	4	37.5	
4	1	42.0	Hykinone 20 mg
	2	39.0	
	3	42.1	
	4	36.2	
	5	39.0	
	6	41.5	
5	1	40.0	Hykinone 20 mg
	2	39.0	
	3	32.0	
	4	30.0	
	5	39.0	
6	1	39.2	Hykinone 20 mg
	2	37.5	
	3	37.0	
	4	37.0	
	5	38.5	

\* Hykinone = 2-methyl-1,4 naphthohydroquinone sodium bisulfite

TABLE 3 (Continued)

Case No	Day	Prothrombin Time of Diluted (12.5%) Plasma	Intravenous Medication
7		<i>sec</i>	
	1	41 6	
	2	45 0	
	3	40 8	Synkayvite* 76 mg.
	4	43 8	Synkayvite 76 mg
	5	41 5	
8	1	41 4	
	2	42 8	
	3	40 4	Synkayvite 76 mg
	4	36 4	Synkayvite 76 mg
	5	37 8	
9	1	35 0	
	2	37 2	
	3	33 8	Synkayvite 76 mg
	4	37 5	Synkayvite 76 mg
	5	37 4	
10	1	38 8	
	2	42 0	
	3	37 2	Synkayvite 76 mg
	4	36 4	Synkayvite 76 mg
	5	44 2	
11	1	40 0	
	2	35 2	
	3	36 2	Synkayvite 76 mg
	4	35 0	
	5	36 0	
12	1	40 0	
	2	36 5	
	3	35 4	Synkayvite 76 mg
	4	40 4	

\* Synkayvite = 2-methyl-1,4 naphthohydroquinone diphosphoric acid ester sodium salt

TABLE 3 (Concluded)

Case No	Day	Prothrombin Time of Diluted (12.5%) Plasma	Intravenous Medication
13		<i>sec</i>	Synkayvite 76 mg
	1	40 8	
	2	43 8	
	3	39 0	
14	4	39 0	Synkayvite 76 mg
	1	44 0	
	2	35 4	
	3	35 0	
	4	32 8	
15	5	43 8	Synkayvite 76 mg
	1	39 2	
	2	40 4	
	3	34 2	
	4	41 4	

When the prothrombin time of whole plasma is estimated, clotting occurs so rapidly that fine alterations are obliterated. The dilution of 12.5 per cent has been used because it has been found by experience that it is highly sensitive, and because the end point in this dilution remains clearly discernible even in the presence of hypoprothrombinemia, such as is encountered after dicumarol medication and in liver disease. Dilution beyond 12.5 per cent and to 8 per cent, although adequate in normal plasma, has been found to be generally unsatisfactory when the prothrombin time is prolonged. In the present series we studied blood plasma of normal or accelerated prothrombin activity. Consequently the dilution of 8 per cent was used to amplify the changes in prothrombin time to a degree greater than that demonstrated in 12.5 per cent plasma. The end point in each case was clearly detectable.

Eleven subjects were given the treatment described above and changes in 8 per cent plasma noted. Seven yielded significant reduction of the prothrombin time while the remaining 4 showed no alteration in the prothrombin values. A statistical analysis of these data follows.

The data were divided into two groups. Group I consisted of the times required for clotting before the administration of vitamin K. Group II was the lowest prothrombin times after the antihemorrhagic quinone was given.

Since the group consists of only 7 cases, the data were treated differently from the series in which 12.5 per cent plasma was studied. The analysis of variance

method was used to compare the groups and the  $t$  value was calculated.<sup>10</sup> The formula used was

$$\frac{(\bar{x} - m)}{S/\sqrt{N}}$$

where  $m$  is the hypothesized mean difference between the groups,  $\bar{x}$  the obtained difference between the groups,  $S$  the standard deviation of the mean difference, and  $N$  the number of cases. We make the initial assumption that there is no differ-

TABLE 4

Case No	Day	Prothrombin Time of 8% Plasma	Intravenous Medication
1	1	63.9	Synkayvite* 76 mg
	2	61.0	Synkayvite 76 mg
	3	57.0	
	4	19.0	
2	1	74.3	Synkayvite 76 mg
	2	74.1	Synkayvite 76 mg
	3	64.4	
	4	47.7	
3	1	71.0	Synkayvite 76 mg
	2	64.0	Synkayvite 76 mg
	3	56.0	Synkayvite 76 mg
	4	48.6	
4	1	71.4	Synkayvite 76 mg
	2	59.9	Synkayvite 76 mg
	3	54.0	
5	1	58.0	Synkayvite 76 mg
	2	52.0	Synkayvite 152 mg
	3	47	
6	1	61.0	Synkayvite 76 mg
	2	58.0	Synkayvite 152 mg
	3	49.0	
7	1	56.5	Synkayvite 76 mg
	2	54.0	Synkayvite 76 mg
	3	46.0	

\* Synkayvite = 2-methyl-1,4 naphthohydroquinone diphosphoric acid ester tetrasodium salt

ence between the two groups (Null Hypothesis).<sup>11</sup> In that case, the hypothesized mean of the difference ( $m$ ) would be zero. The  $x$  value is obtained by summing the difference between the individual cases in group I and group II and dividing by

the number of cases The standard deviation of the mean so obtained is calculated in the usual way

## RESULTS

Case No	Group I	Group II	Diff
1	63 9	49 0	14 8
2	74 3	47 7	26 6
3	71 0	48 6	22 4
4	71 4	54 0	17 4
5	58 0	47 0	11 0
6	61 0	48 0	13 0
7	56 5	46 0	10 5
		Mean Diff	= 16 53
		S Diff	= 9 66
		<i>t</i>	= 4 5

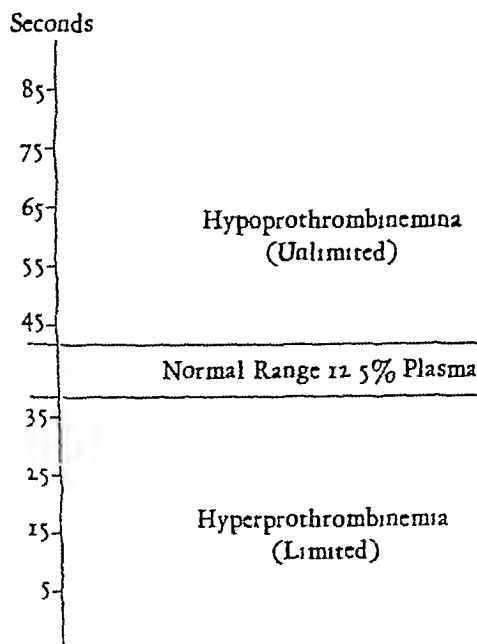
Table I Calculating the *t* value to compare the difference between time required for clotting before and after the administration of vitamin K. The *t* value obtained (4.5) was compared to the value of *t* at the 1 per cent level of confidence for 6 degrees of freedom.<sup>12</sup> The obtained *t* value of the 1 per cent level was 3.707, and this latter would be expected to occur only once in a hundred samples if *m* really were equal to zero difference between the two groups.

## CONCLUSIONS

The null hypothesis in this group of observations must be abandoned. The difference between the two groups is highly significant at the 1 per cent level of confidence.

## DISCUSSION

The literature reflects a total lack of appreciation of the difference between changes in the hyper range and those occurring in the direction of hypoprothrombinemia. To illustrate this, the following diagram is presented.





Reduction in prothrombin time below the normal range (hyperprothrombinemia) meets with resistance of increasing magnitude as it progresses. Opposed to this is the prolongation of the prothrombin time, which proceeds with greater and greater ease as the degree of hypoprothrombinemia becomes more extensive. For example, a small dose of dicumarol will extend the prothrombin time from the hypoprothrombinemia level of sixty to eighty seconds, whereas a large dose is required to increase it from the original normal of forty seconds to sixty seconds. In contrast to this, it requires an agent of high potency to reduce the prothrombin time from the normal of forty to the hyperprothrombinemia level of thirty-six seconds, and to decrease it further meets with resistance of such proportions that it is accomplished with the greatest difficulty. Obviously, it is difficult to reproduce these changes by using methods of prothrombin estimation which employ whole plasma because clotting occurs so speedily that the fine changes are obliterated. Only by amplifying the differences by plasma dilutions can these changes, significant despite their relatively low magnitude, be detected.

It is important to note the difference in the pattern of hyperprothrombinemia as it arises spontaneously in certain clinical conditions (thrombosis, frost-bite, gangrene) and that induced by vitamin K. The clinical form continues for extended periods, whereas that induced by the antihemorrhagic substance is ephemeral. It appears that an additional mechanism enters the process when intravascular coagulation takes place, and it suggests at least a partial explanation for the tendency to multiple thrombosis.

It is not known whether or not in man other components of the blood which enter into the mechanism of coagulation vary in like manner after the giving of vitamin K. Work is now in progress in this laboratory to learn whether the fibrinogen and the thrombocytes alter under these conditions. It has been our experience in man that when we employed human fibrinogen in a concentration of 0.3 per cent as a diluent in place of normal isotonic saline that the values obtained, especially when diluted plasma was studied, were highly variable in normal as well as in pathological cases. Our original technic at the same time yielded results in serial estimations which varied within a very narrow range. In any event, if our subsequent findings reveal changes in the companion components of the coagulation system after vitamin K, then the present conception of the role of vitamin K will have to be extended.<sup>13</sup>

It is our belief that the behavior of the prothrombin-producing mechanism as revealed by the data presented in this communication is an expression of functional limitation and not a mathematical difficulty of depression to zero. The apparent resistance to lowering of the prothrombin time of the plasma below the existing resting level by experimental means should be viewed as a characteristic of this function of the liver. We have observed identical behavior in prothrombin deficiency states also. Thus, in the hypoprothrombinemia accompanying cirrhosis of the liver, serial estimations of prothrombin time have been demonstrated to be remarkably constant within narrow limits over periods of many weeks. Furthermore, when the prothrombin activity was altered by experimental means in these cases, the prothrombin time returned to approximately the initial value when

recovery occurred.<sup>6</sup> The indications seem to be that the liver delivers its maximum supply of prothrombin under the prevailing conditions, and that when the tempo can be accelerated at all it continues at the new rate for transitory periods only.

It is noteworthy that the changes in the direction of hyperprothrombinemia occurred in only about half of the cases, despite the fact that all exhibited normal resting levels of prothrombin activity. The mechanism suggested is that vitamin K activates a substrate, and that variations in the capacity of different livers to store this substrate might account for the different responses indicated above.

The greater magnitude of the change exhibited by increased dilution of the 8 per cent plasma as compared with 12.5 per cent supports our contention that the shift in the direction of hyperprothrombinemia is functional and not an apparent change produced by mathematical manipulation of the data.

### CONCLUSIONS

Reduction of the prothrombin time of diluted (12.5 per cent and 8 per cent) plasma below normal occurred in man with normal liver function following parenteral administration of large doses of menadione derivatives. In each case, where the increase was demonstrable, it continued for transitory periods only, lasting twenty-four to forty-eight hours.\*

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\*In the bio-assay employed in these studies, prothrombin activity is estimated. Whether this may be interpreted in terms of prothrombin concentration of the plasma remains to be determined. Possibly a repetition of the work using the two stage method might aid in answering the question.

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# A MODIFICATION OF THE WAUGH-RUDDICK TEST FOR INCREASED COAGULABILITY OF THE BLOOD, AND ITS APPLICATION TO THE STUDY OF POSTOPERATIVE CASES

By SEYMOUR B SILVERMAN, M D \*

IN 1944, Waugh and Ruddick<sup>1</sup> reported a new test for increased coagulability of the blood, based on controlled deceleration of the clotting mechanism through the use of heparin. While it was appreciated that temperature affected the test, the original work was done at a time when room temperature was constant (i.e., during the winter months), so that special precautions in this direction were unnecessary. Later Whittaker,<sup>2</sup> working during the summer months, when room temperature fluctuations were present, experienced some difficulty in obtaining duplicate curves, and worked out in detail the facts concerning the effect of temperature on the test. She found that between 20 and 35 degrees Centigrade, increase in temperature caused a decrease in clotting time. This stressed the fact that comparison of the blood coagulation curves in different individuals, or in the same individual at different times, is possible only if the tests are conducted at the same temperature.

Application of the test to clinical material<sup>3</sup> revealed an increased coagulability of the blood under a wide variety of circumstances. These include pneumonia, empyema, peritonitis, and other acute infectious processes following hemorrhage after surgical operation, etc. More recently, Ogura and colleagues<sup>4</sup> have used the Waugh-Ruddick test to study changes in blood coagulation following coronary thrombosis. They found that in 27 cases, 77.8 per cent showed a decreased coagulation time. This was usually evident by the second or third day following the thrombotic incident, and lasted to about the seventeenth day. Acceleration was prolonged beyond the third week in a few cases, but in every instance, the clotting mechanism was indistinguishable from normal after the fourth week.

The present report deals with (1) a proposed modification of the test, and (2) the application of the modified test to the study of postoperative blood coagulability.

## A MODIFICATION OF THE WAUGH-RUDDICK TEST

Using the original method of Waugh and Ruddick,<sup>1</sup> blood coagulation studies were carried out on 43 student volunteers. The average curve for this group is shown in figure 1 along with the average curve as determined by the original investigators. It will be seen that although the curves have the same general form, the actual values obtained differ greatly in the two series. There appear to be

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two chief reasons for this, viz, (1) the heparin employed was of different batches and thus possibly of somewhat different potencies, and (2) the endpoint in each tube is not sharply defined, and consequently there arises the question of interpretation of results. These and other reasons for modifying the test can be summarized as follows:

1. To shorten the time needed to complete a test

2. To perform the test under controlled temperature conditions

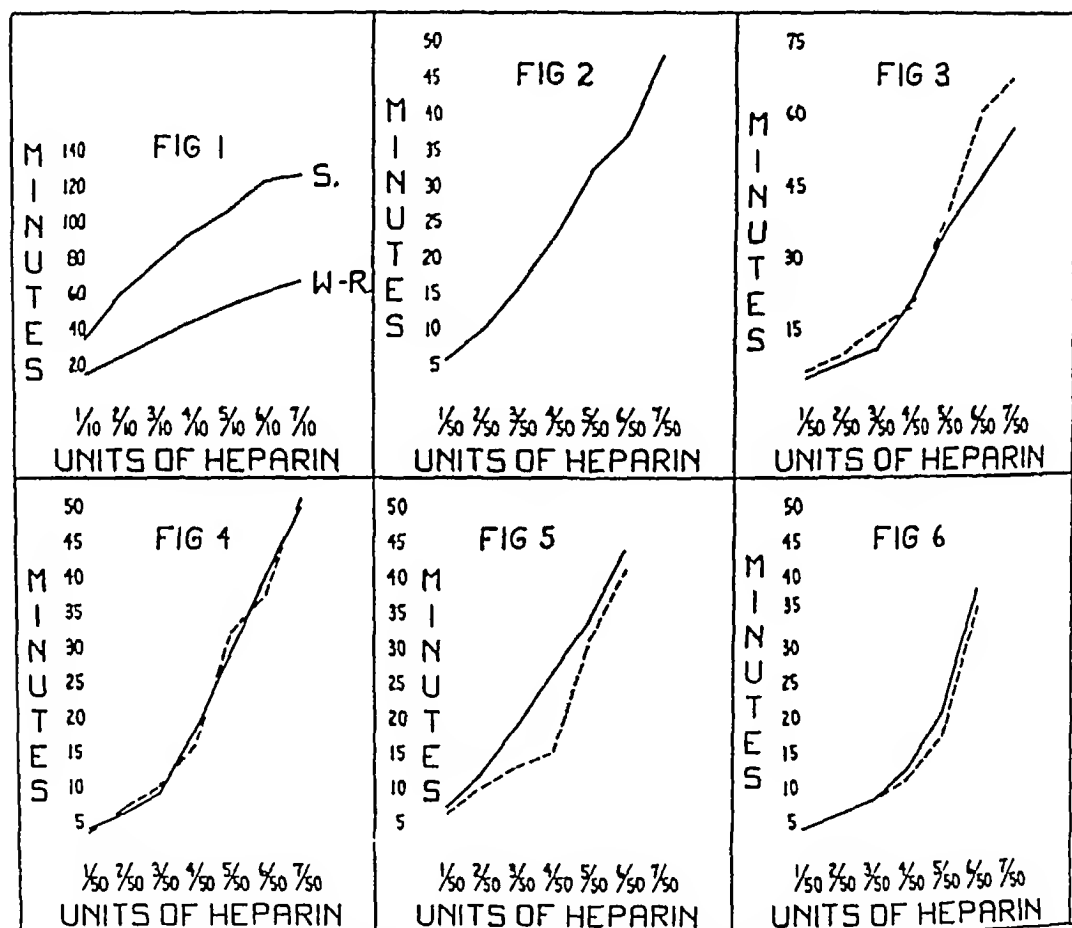


FIG 1 Original Waugh-Ruddick test. Average curves obtained by Waugh and Ruddick, and the author.

FIG 2 Modified Waugh-Ruddick test. Average curve obtained from 36 normal specimens of plasma.

FIGS 3-6 Duplicate curves on normal individuals.

3. To obtain a sharper and more definite endpoint in all tubes

4. To reduce the amount of blood needed to perform the test

Details of the modified test follow:

**Solutions Used** (1) Heparin stock solution containing 2 units of heparin per cc of saline. The Connaught Laboratory preparation, having a potency of 1000 units per cc, is employed. Six-tenths of a cc of heparin are added to 300 cc of physiological saline, and thoroughly mixed. This stock solution is used in making up the various subdivisions of heparin, and can be kept in the icebox when not needed.

(2) Subdilutions of heparin are made up as shown in table 1. These are kept in small glass-stoppered bottles with large mouths (capacity 60 cc). The potency of the heparin is maintained by keeping these solutions in a refrigerator.

(3) 0.1 M sodium oxalate: 1.34 Gm. of sodium oxalate are dissolved in 100 cc. of distilled water.

(4) 0.01 M calcium chloride: 0.11 Gm. of anhydrous calcium chloride and 0.42 Gm. of sodium chloride are dissolved in 100 cc. of distilled water.

*Preparation of Test Tubes* Eight Wassermann tubes (100 x 13 mm.) (previously cleaned in potassium-dichromate-sulphuric acid solution, rinsed out in hot water and distilled water, and dried in an oven) are placed in a suitable rack, and are numbered from 1 to 8. To each tube are added 0.2 cc. of 0.01 M calcium chloride and 0.1 cc. of the heparin subdilution from the correspondingly numbered glass-stoppered bottle. The tubes are corked and set aside.

TABLE 1 — *Preparation of the various subdilutions of heparin, showing the amounts of physiological saline and heparin stock solution used and the resultant heparin concentration in each case*

Bottle	Stock Soln	Saline Soln	Resultant Heparin Concentration
	cc	cc	unit per 0.1 cc
1	0	50	0
2	5	45	1/50
3	10	40	2/50
4	15	35	3/50
5	20	30	4/50
6	25	25	5/50
7	30	20	6/50
8	35	15	7/50

*Preparation of Plasma to be Tested* By venipuncture, using a dry 20 to 30 cc. graduated Luer syringe and a large gage (No. 18) needle, 4.5 cc. of blood are drawn and mixed immediately and thoroughly with 0.5 cc. of 0.1 M sodium oxalate by repeatedly inverting the stoppered tube. The blood is centrifuged at the rate of 1000 r.p.m. for five minutes, and during this time the rack containing the prepared tubes is placed in a water bath at 37.5 degrees C. This assures the tubes being at body temperature for the test.

After centrifuging for five minutes, the plasma is removed from the packed cells, and 0.1 cc. is added to each of the eight tubes in a water bath. The actual test now begins.

*The Test Proper* As soon as the plasma has been added to the heparinized tubes, they are agitated slightly to ensure complete mixing of the fluid and the plasma. A stop watch is started when plasma has been added to the last tube.

Starting at about two and a half minutes, the first tube is gently partially withdrawn from the rack, and the plasma is noted for clotting. If this has not occurred, the tube is replaced and examined at half-minute intervals until a complete firm clot has formed. The time is noted. The same procedure is then carried on for tube

2, then tube 3, etc until coagulation has occurred in all tubes The results are plotted on graph paper, the coagulation time against the amount of heparin in each tube

*Some Observations Using the Modified Test* Tests were done on 36 samples of normal plasma The mean, standard deviation, maximum and minimum values for each tube are given in table 2 The average curve is shown in figure 2 As is to be expected, some individual variation is seen, but this is of the same order as that described in the original Waugh-Ruddick method That the test is reliable in any given case was shown by doing checks on the same plasma samples (figs 3, 4, 5, 6)

*Discussion Regarding the Modified Test* The modification proposed is in reality a combination of Quick's method of determining the clotting time of recalcified plasma<sup>6</sup> and the Waugh-Ruddick test<sup>1</sup> As stated by Chargaff, Bancroft and Stanley-Brown, working on methods for the measurement of inhibition of clotting by various substances including heparin, such a method "can only have the accuracy of

TABLE 2 — *Modified Waugh Ruddick Test*

The mean, standard deviation, maximum and minimum values obtained from 36 specimens of normal plasma

Tube	Minimum	Mean	Maximum	Standard Deviation
	<i>mins</i>	<i>mins</i>	<i>mins</i>	
1	2 5	3 46	5 0	0 5
2	4 0	6 46	9 5	1 3
3	5 8	10 8	22 0	3 45
4	8 2	16 8	39 0	7 18
5	12 0	23 8	50 0	9 64
6	15 0	33 1	55 0	10 9
7	21 5	38 2	62 5	10 2
8	22 7	49 5	69 0	12 3

the biological test, and not that of a quantitative chemical procedure The chief reason is the fact that the endpoint, namely the formation of a clot, is comparatively ill-defined "6 The average curve (fig 2) is simply that—an average, and while some tests vary considerably from this, the results obtained in any one case are fairly constant The use of plasma instead of whole blood has rendered the endpoint easier to determine, but other factors equally as important must be taken into consideration As Chargaff<sup>6</sup> points out, to be successful in studying the action of inhibitors on plasma coagulation the following must be considered

1 The reaction volume must be kept constant, as the numerous substances contained in plasma respond to dilution in different ways The addition, to a series of plasma samples, of increasing amounts of inhibitor solution leads to discordant results

2 Extreme care must be taken to disturb the plasma as little as possible Attempts to increase the precision of the endpoint may adversely affect its accuracy Plasma which coagulates in the presence of an inhibitor in general gives rise to clots that are very soft, and may easily be broken up beyond recognition if shaken before their formation is complete

3 The period of time over which the determination of inhibitor activity takes place should not be too extended, as the clotting properties of plasma may change quite considerably with time

It is felt that the modification outlined fulfills these conditions, and retains the advantages of the Waugh-Ruddick test while avoiding some of the disadvantages

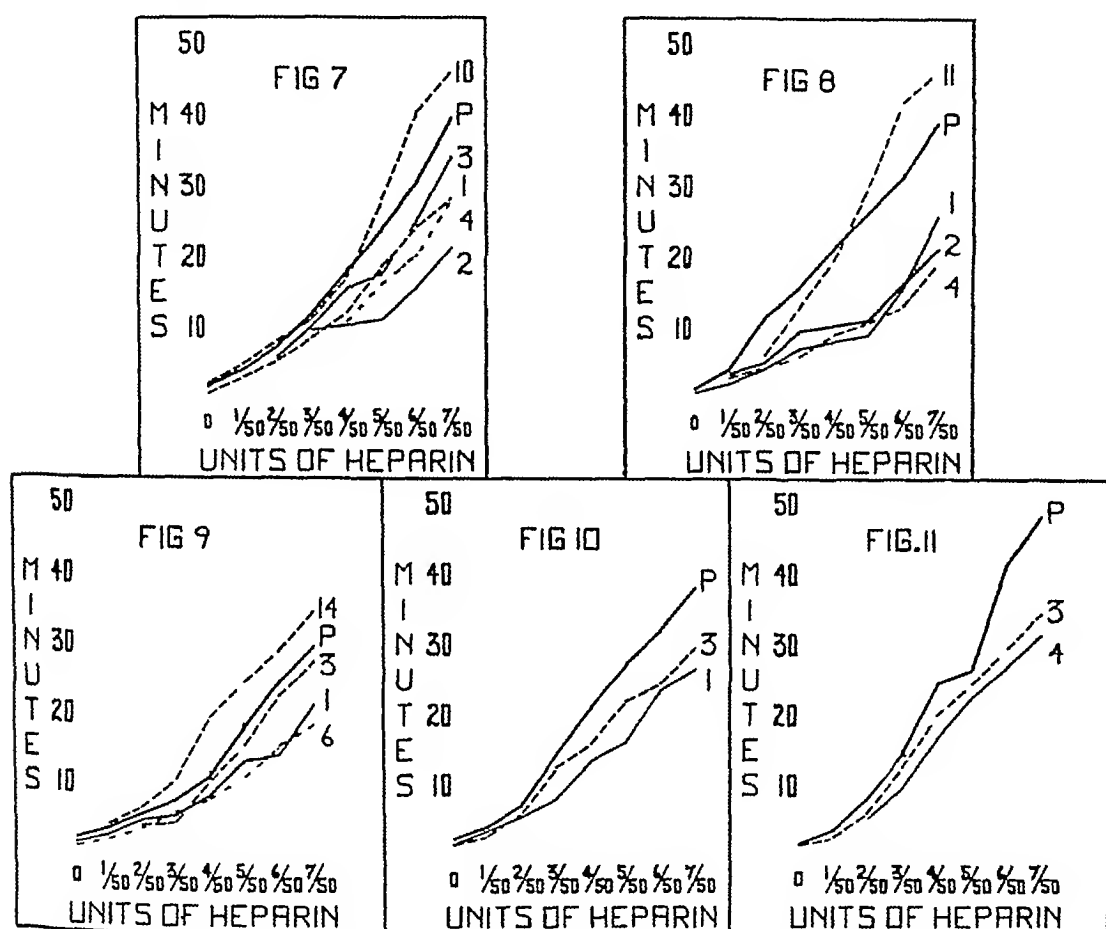


FIG 7 Average curves preoperatively (P), and 1, 2, 3, 4, and 10 days postoperatively

FIG 8 Case 1 Right inguinal herniorrhaphy Curves obtained preoperatively and 1, 2, 4, and 11 days postoperatively Note the increased coagulability postoperatively, present on the first day Coagulability back to normal by the eleventh day

FIG 9 Case 2 Right inguinal herniorrhaphy Curves preoperatively and 1, 3, 6, and 14 days postoperatively Increased coagulability is seen within 24 hours after operation

FIG 10 Case 3 Appendectomy The increased coagulability seen postoperatively is indicated by a clockwise shift of the curves

FIG 11 Case 4 Appendectomy Clockwise shift of coagulability curves postoperatively, i.e., increased coagulability

#### APPLICATION OF THE MODIFIED TEST TO THE STUDY OF POSTOPERATIVE BLOOD COAGULABILITY

Using the above test, it was decided to study the affect of operation on the coagulability of the blood Waugh and Ruddick<sup>3</sup> using their test in a wide variety of conditions, applied it to several patients undergoing operation They found a marked increased coagulability after operation, but did not investigate the time



at which it first appeared nor its duration. They state "It would be interesting if finer analysis of this change were studied in order to demonstrate the exact time at which increased coagulability occurs." That is exactly what the present study attempts to accomplish.

In this investigation, tests were performed on 9 patients admitted to the Royal Victoria Hospital for operation. A special effort was made to use patients with no acute illness or any other condition likely to affect the coagulability of the blood. Eight of these were for appendectomy or herniorrhaphy. The ninth was a gastrectomy. All operations were performed under spinal anesthesia. Coagulation tests were done preoperatively and as often postoperatively as possible, commencing the day after operation. The average curves obtained preoperatively and on the first, second, third, fourth, and tenth days postoperatively are shown in figure 7. Some individual records are shown in figures 8-11. In figures 7-9, not all the observed days are recorded, to avoid complicating the graphs. Figures 10 and 11 are complete. Some illustrative cases follow.

*Case 1* Mr. P., age 56, admitted for a right inguinal herniorrhaphy. It will be noted that the postoperative coagulability curves are below the preoperative level immediately after operation (i.e., within 24 hours), and for several succeeding days. The coagulation time slowly returns to normal, and is within normal limits by the eleventh day (fig. 8).

*Case 2* Mr. C., age 49, admitted for a right inguinal herniorrhaphy. The curves in this case are much like those in case 1. There is a marked increase in blood coagulability postoperatively, with a slow return to normal, in this case by the fourteenth day (fig. 9).

*Case 3* Mrs. C., age 37, admitted for appendectomy. The relatively short stay in hospital allowed of only three curves being done. Here again, the increased coagulability postoperatively, indicated by a clockwise shift of the curves, is seen (fig. 10).

*Case 4* Mrs. D., age 29, admitted for appendectomy. The story is much the same as in case 3. See figure 11 for coagulability curves.

## DISCUSSION

The average curves (fig. 7) indicate that there is a definite increased coagulability of the blood postoperatively. This begins within 24 hours after operation, and may last, in varying degrees, for a week or more. There does not appear to be a definite order in which the coagulability changes from day to day, but this is hardly to be expected, as the test must of necessity be a qualitative rather than a precisely quantitative affair. In all cases where hospitalization was long enough to permit observations, the coagulation time was normal at the end of two weeks.

Quick<sup>7</sup> points out that "thromboplastin is the trigger-substance in the coagulation process. It initiates and determines the speed of the reaction." If this is so, then the decreased coagulation time seen postoperatively can be explained by an increase in the amount of available thromboplastin. There are two reservoirs of this material, namely the platelets and the tissue juices. Let us consider each of these in turn. Hueck<sup>8</sup> in 1926 first demonstrated the presence of a postoperative thrombocytosis. This has since been confirmed by many other workers.<sup>9-15</sup> All agree that this increase in platelets occurs about the sixth or seventh postoperative day. It therefore cannot explain the increased coagulability of the blood seen within 24 hours after operation.

On the other hand, an increase in the circulating thromboplastin, presumably derived from damaged tissue in the operative area, is a much more likely explanation. This view has been favored by Pickering and Mathur,<sup>16</sup> Dougal<sup>17</sup> and others, and is supported by observations that the blood urea and polypeptides are increased postoperatively.<sup>17</sup> Snell<sup>18</sup> and Bancroft<sup>10</sup> point out that after operations on obese patients there may be an increased liberation of thromboplastic lipoid substances such as cephalin, due to extensive areas of fat invaded. Waugh and Ruddick<sup>3</sup> showed that the addition of thromboplastin to plasma caused a clockwise shift of the coagulability curves, such as has been shown to occur postoperatively. It would appear that the mechanism is the same in both instances.

#### SUMMARY

1. A modification of the Waugh-Ruddick test for increased coagulability of the blood is described, which employs the use of recalcified plasma in the place of whole blood.

2. Using this modification, studies were carried out on a series of patients undergoing operation. It was found that there was an increased coagulability of the blood present within 24 hours following operation, and that this condition lasted for a week or so. In all cases, the coagulability had returned to normal by the end of two weeks.

3. It is felt that the change is due to an increase in the circulating thromboplastin, presumably derived from damaged tissue in the operative area.

#### ACKNOWLEDGMENT

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# IN VITRO STUDY OF BONE MARROW III ERYTHROPOIESIS IN VITRO OF STERNAL MARROW FROM CASES OF PERNICIOUS ANEMIA AND LYMPHATIC LEUKOSIS UNDER THERAPY

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PREVIOUS investigations by one of us (C M P ) have demonstrated the possibility of keeping a suspension of bone marrow alive in vitro for several hours for studies on erythropoiesis.<sup>3</sup> In the present investigation we have utilized this technic to examine the erythropoietic activity of marrow from normal persons and from cases of pernicious anemia and lymphatic leukosis before and after specific treatment

## TECHNIC

The apparatus employed has been described in detail in a previous communication.<sup>3</sup> The bone marrow was kept alive between two concentric collodion membranes, and a continuous flow of nutritional fluid was sent through the central chamber, thus permitting a constant composition of the nutritional medium within it, in spite of the diffusion taking place through the membrane out into the suspension of marrow. To remove waste products the Locke's fluid in the peripheral chamber was frequently renewed. The temperature was kept constant at 37°C by means of a water bath, and a continuous current of carbogen was sent through the chamber.

Marrow was obtained by puncture of the sternum and aspiration of 0.5 cc. of marrow. Sodium citrate 3.5 per cent was added in equal parts, and the cells separated from the fluid by centrifugation. With a pipet the intermediary layer of cells was removed for cultivation and brought to a concentration of about 30,000 cells per cu. mm. in Locke's fluid containing 1 per cent of the corresponding serum. The serum to be examined was suspended in Locke's fluid to a concentration of 2 per cent and used as nutritional medium.

It was necessary to start cultivation as soon as possible after the sternal puncture, and as no absolute sterility of the apparatus is obtainable the cultivation period was limited to five or six hours.

Experiments on leukotic marrow were carried out simultaneously in a set of four parallel apparatus, kept in the same water bath. In these cases the first apparatus tested normal marrow against its own serum as nutritional medium, the second chamber contained normal marrow with leukotic serum, the third, leukotic marrow nourished with normal serum, the fourth tested leukotic marrow against its own serum. Each experiment thus contained satisfactory controls.

The erythropoietic activity was expressed by the number of non-nucleated cells produced during one hour per nucleated red cell present before cultivation. The determination of this value may be illustrated by an example from the cultivation of marrow from Case 2.

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		1 Start	2 3 hours	3 5 hours
Total cell count per cu mm	A	42880	44040	44720
Nucleated cell count per cu mm	B	3640	3480	3360
Total non-nucleated cells mm <sup>3</sup>	C = A - B	39240	40560	41360
Non-nucleated cells produced mm <sup>3</sup>	D = C <sub>1</sub> - C <sub>2</sub>		1320	2120
Immature red cells per mm <sup>3</sup>	E = % of B	6% = 218	6% = 209	5% = 168
Dc produced per nucleated R B C per hour	$\Gamma = \frac{DX_2}{(E_1 - E_2) \times \text{hours}}$		2.03	2.21

## NORMAL INDIVIDUALS

It is a weakness of the method that no fixed standard for erythropoietic activity was available. In every case control experiments must be carried out on normal bone marrow planned as the cross experiments to be described. Thus it was im-

TABLE 1—Normal Marrows and Sera Erythropoietic Activities

I	Normal Serum A	Normal Serum B	II	Normal Serum C	Normal Serum D	III	Normal Serum E	Normal Serum F
Normal Marrow A	5 41	4 50	Normal Marrow C	5 17	4 43	Normal Marrow E	4 98	4 18
Normal Marrow B	4 25	5 04	Normal Marrow D	4 51	5 29	Normal Marrow F	4 08	4 85

IV	Normal Serum G	Normal Serum H	V	Normal Serum I	Normal Serum K
Normal-Marrow G	5 09	4 42	Normal Marrow I	4 83	4 05
Normal Marrow H	4 16	4 78	Normal Marrow K	4 15	5 05

portant to know the values for erythropoietic activity of normal marrow. Examinations of marrows from 31 persons were carried out in single experiments with the following results. Average activity was  $5.13 \pm 0.26$ , the maximum value found was 5.88, and the minimum 4.67. Five cross experiments on ten marrows and sera among the 31 are recorded in table 1.

Using the values given above indicating erythropoietic activity we worked out the following ratios for each cross-experiment and the average for all

	I	II	III	IV	V	
1 $\frac{\text{Marrows B, D, F, H, K} + \text{Sera B, D, F, H, K}}{\text{Marrows A, C, E, G, I} + \text{Sera A, C, E, G, I}}$	0.93	1.02	0.97	0.94	1.04	Average 0.98
2 $\frac{\text{Marrows B, D, G, F, K} + \text{Sera A, C, E, G, I}}{\text{Marrows A, C, E, G, I} + \text{Sera A, C, E, G, I}}$	0.79	0.87	0.82	0.82	0.86	0.83
3 $\frac{\text{Marrows A, C, E, G, I} + \text{Sera B, D, F, H, K}}{\text{Marrows A, C, E, G, I} + \text{Sera A, C, E, G, I}}$	0.83	0.86	0.84	0.87	0.84	0.85
4 $\frac{\text{Marrows B, D, F, H, K} + \text{Sera A, C, E, G, I}}{\text{Marrows B, D, F, H, K} + \text{Sera B, D, F, H, K}}$	0.85	0.85	0.84	0.87	0.82	0.85
5 $\frac{\text{Marrow A, C, E, G, I} + \text{Sera B, D, F, H, K}}{\text{Marrows B, D, F, H, K} + \text{Sera B, D, F, H, K}}$	0.89	0.84	0.86	0.92	0.80	0.86

It appears from these figures that as soon as a marrow is nourished on serum from another person erythropoietic activity is reduced to about 85 per cent of the activity between corresponding marrow and serum, but the activity in such a heterologous system is the same whichever marrow-serum combination is employed

In subsequent experiments, similar ratios were set up to calculate the relative effects of normal and pathologic sera upon normal and pathologic marrows. Numbered according to the schema above, these are as follows

		Normal Average
1	$\frac{\text{marrow B} + \text{serum B}}{\text{marrow A} + \text{marrow A}}$	0 98
2	$\frac{\text{marrow B} + \text{serum A}}{\text{marrow A} + \text{serum A}}$	0 83
3	$\frac{\text{marrow A} + \text{serum B}}{\text{marrow A} + \text{serum A}}$	0 85
4	$\frac{\text{marrow B} + \text{serum A}}{\text{marrow B} + \text{marrow A}}$	0 85
5	$\frac{\text{marrow A} + \text{serum B}}{\text{marrow B} + \text{serum B}}$	0 86

The authors realize that computations carried out in accordance with usual conceptions of the validity of standard deviations for countings of cells in fluid media might find our results within the limits of standard deviations. However, after studies by G. Rasch on results obtained by Ruth Plum, it must be accepted that such enumerating can be carried out with far greater accuracy than hitherto assumed.<sup>3, 4</sup> Finally the influence of therapy on our results prevent us from regarding them as merely accidental.

#### SIMPLE ANEMIA

Cross experiments were carried out in 2 cases of slight anemia

*Case A* Woman, aged 32. Menses normal, last birth six months earlier. Now tired, irritable and nervous. No abnormal somatic findings, no enlargement of lymph nodes, no goiter. Hb 74 per cent, erythrocytes 4.7 M, color index 0.78. Differential counts of peripheral blood and bone marrow showed normal values.

	Serum Anemic	Serum Normal	Erythropoietic activity on alien serum as per cent of activity on own serum
Anemic Marrow	4 88	4 48	92
Normal Marrow	4 40	5 45	81
			Normal Average
1	$\frac{\text{Path Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 88	0 98
2	$\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 82	0 83

		Normal Aver	
3	$\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 81	0 85
4	$\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Path Marrow} + \text{Path Serum}}$	0 92	0 85
5	$\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Path Marrow} + \text{Path Serum}}$	0 90	0 86

*Case B* Male, aged 28 For the last years very irregular meals Pronounced fatigue and slight dyspepsia combined with headache No pathologic somatic findings Gastric analysis normal Hb 79 per cent, Erythrocytes 4 4 M, Color Index 0 84 Differential count of peripheral blood gave normal values The sternal marrow showed fairly numerous cells of the erythropoietic system (38 per cent)

	Anemic Serum	Normal Serum	Erythropoietic activity on alien serum as per cent of activity on own serum
Anemic Marrow	4 69	4 17	89
Normal Marrow	4 37	5 02	87

		Normal Aver	
1	$\frac{\text{Path Marrow} + \text{Path Serum}}{\text{Normal Marrow} + \text{Norm Serum}}$	0 93	0 98
2	$\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 83	0 83
3	$\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 87	0 85
4	$\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Path Marrow} + \text{Path Serum}}$	0 89	0 85
5	$\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Path Marrow} + \text{Path Serum}}$	0 94	0 86

Thus, marrows from two cases of simple anemias did not show any pronounced deviation from normal marrows

#### PERNICIOUS ANEMIA TREATED

*Case 1* Woman, aged 44 For about six months, the patient suffered from constipation, increasing fatigue, pains in the tongue and epigastrium Laboratory showed gastric achylia On May 15, 1946 the blood values showed Hb 62 per cent, R B C 2 0 M, color index 1 48, icterus index 7 A differential count of the marrow showed blasts 4 4 per cent, promyelocytes 0 8 per cent, myelocytes 14 4 per cent, metamyelocytes 14 8 per cent, bands 34 per cent, polys 15 2 per cent, monos 0 2 per cent, lymphos 10 2 per cent, plasma cells 0 6 per cent, normoblasts 19 4 per cent, megaloblasts 16 2 per cent

Following treatment with liver extract, within sixteen days the erythrocytes numbered 3 8 million, the hemoglobin was 83 per cent, and the color index was 1 08. The reticulocytes had reached a maximum of 25 7 per cent.

TABLE 2.—*Case 1*

	5/15/46 Pernicious Anemia Serum I (before treatment)	Pernicious anemia		Erythropoietic ac- tivity on alien serum as per cent of activ- ity on own serum
		6/1/46 Serum II (after treat and fall in reticulocyte)	Normal Serum a, b	
Marrow I	2 91		a 4 42	152
Marrow II		4 38	b 5 05	115
Normal Marrow a	3 12		a 4 67	67
Normal Marrow b		4 18	b 5 23	80

Table 2 lists the results of cross experiments using this patient's marrow and various sera.

	I	II	Norm Aver
1 $\frac{\text{Path Marrow} + \text{Path Serum}}{\text{Normal Marrow} + \text{Norm Serum}}$	0 62	0 84	0 98
2 $\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 95	0 96	0 83
3 $\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0 67	0 80	0 85
4 $\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Path Marrow} + \text{Path Serum}}$	1 52	1 15	0 85
5 $\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Path Marrow} + \text{Path Serum}}$	1 08	0 95	0 86

Figures 1 and 2 show that the erythropoietic activity of the pathologic marrow when nourished on normal serum not only did not drop by 15 per cent on application of the alien serum but even exceeded normal erythropoiesis by about 50 per cent (Line 4). The pathologic serum, however, failed to keep a normal alien marrow at 85 per cent of its production with its own serum, and reached a level of only about 67 per cent (Line 3). Both these deviations tended to disappear after treatment.



## LYMPHATIC LEUKOSIS TREATED WITH URETHANE

Shortly after the publication by Paterson, Haddow, Ap Thomas, and Watkinson on the treatment of leukemia with urethane,<sup>1</sup> we began investigations of the effect of such treatment, both clinically and by means of the technic here described. Technical difficulties prevented corresponding studies on the leukopoietic activity of marrow because of the morphologic similarity of different cells in the early development of the white cell series. Cultivation of marrow was carried out before, during, and after treatment with urethane, which in these cases was supplemented

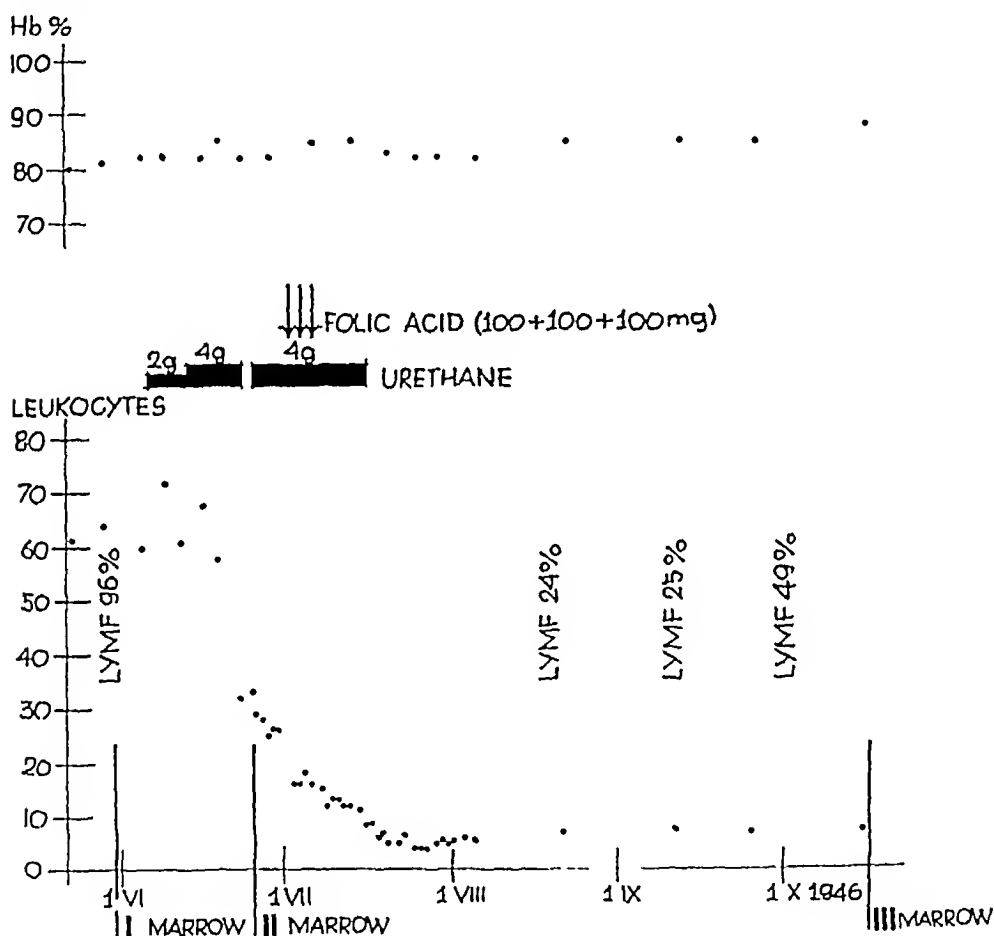


FIG 1 Case 2. Leukosis lymphatica

with folic acid, and which gave results in full conformity with those obtained by Paterson and her colleagues.

*Case 2* (Figure 1) Woman, aged 66. For a few months, there was increasing loss of strength with expectoration and slight rise of temperature. She was admitted to the hospital, and a diagnosis was made of febrile bronchitis, obesity and osteoarthritis of the knee joints. There was no enlargement of lymph nodes, liver, or spleen. The white cell count was 64,000 with 90 per cent lymphocytes. Sternal marrow showed 70 per cent lymphocytes and the case was diagnosed as lymphatic leukemia. Urethane was given in daily doses of 2 to 4 grams (146 grams in forty days) and was followed by a fall in leukocytes from 60,000 to 70,000 to less than

20,000 in four weeks. After treatment for another two weeks the white cell count was below 10,000. At the beginning of treatment 90 per cent of the white cells were lymphocytes, but ten to eleven weeks later lymphocytes amounted to 25 per cent of the white cells.

At one time folic acid was added to the treatment in the hope of stimulating erythropoiesis, 300 milligrams being given in the course of three days. The fall of the white cell count seemed uninfluenced by this medication. The hemoglobin level was fairly constant at about 80 to 85 per cent.

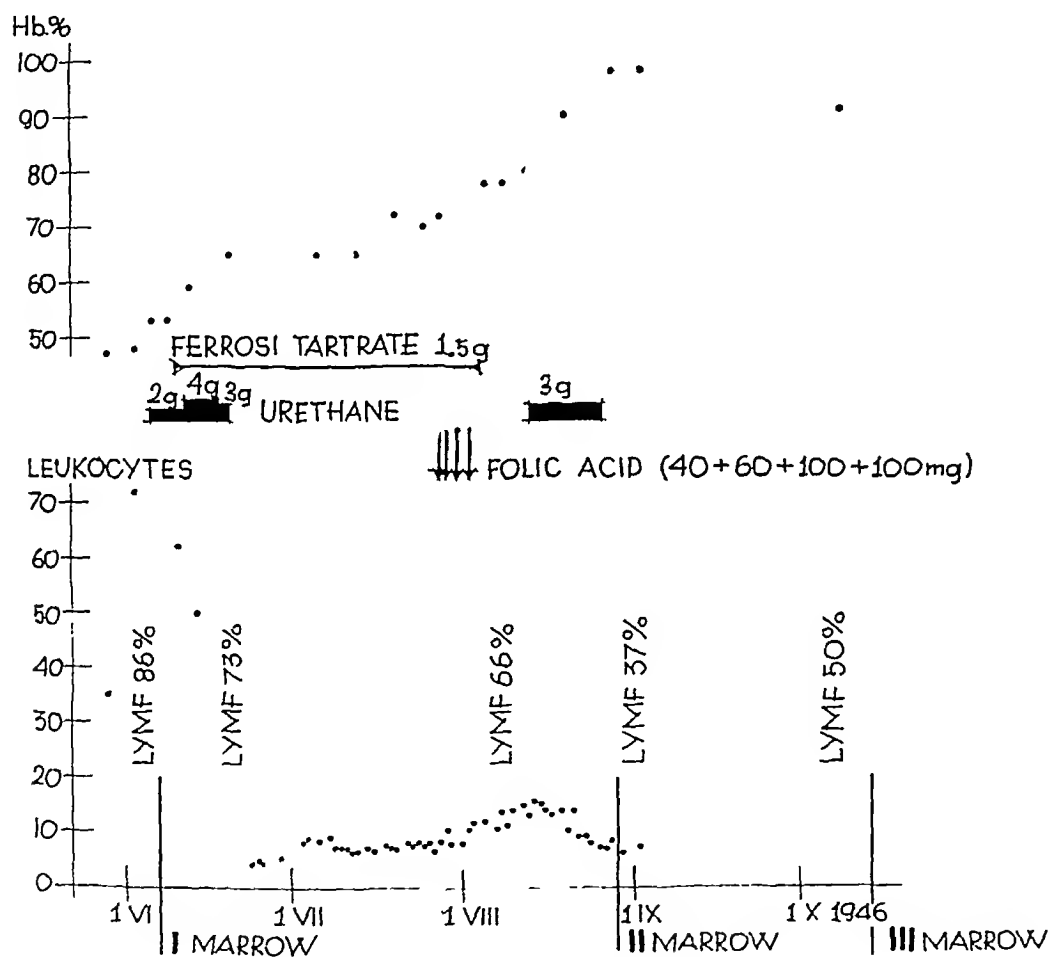


FIG 2 Case 3 Leukosis lymphatica

Three months after the end of treatment the white count was still normal and the patient was feeling well.

**Case 3** (Figure 2) Woman, aged 43. Since adolescence the cervical lymph nodes had been enlarged, varying in size but with no other symptoms. At about 18 she had been treated with roentgen rays, and at 30 by incision of suppurating lymph nodes.

For four years the patient had had increasing loss of strength and weight, progressive enlargement of lymph nodes, sweating, itching of the skin, diarrhea, dry cough, and a tendency to edema of the ankles. Several febrile periods had been

treated as pneumonias In the hospital she was found to have an anemia (Hb 48 per cent), and enlargement of the lymph nodes to the size of an egg, in the neck,

TABLE 3—Case 2

	Serum I, before treatment, 5/31/46	Serum II, during treatment, 6/25/46	Serum III, after treatment, 10/17/46	Normal Serum a, b, c	Erythropoietic activity on alien serum as per cent of activity on own serum
Marrow I	2 12			a 3 12	147
Marrow II		3 97		b 4 67	117
Marrow III			4 93	c 5 03	102
Normal Marrow a	3 04			a 4 77	64
Normal Marrow b		4 17		b 4 87	87
Normal Marrow c			4 74	c 5 27	90

TABLE 4—Case 3

	Serum I before treatment 6/7/46	Serum II, during treatment, 8/29/46	Serum III, on readmission, 10/14/46	Normal Serum a, b, c	Erythropoietic activity on alien serum as per cent of activity on own serum
Marrow I	3 13			a 4 32	138
Marrow II		3 63		b 4 58	126
Marrow III			4 20	c 5 15	120
Normal Marrow a	3 49			a 5 21	67
Normal Marrow b		3 38		b 4 83	70
Normal Marrow c			4 56	c 5 36	85

the axillae and the groins The spleen was enlarged 4 to 5 cm below the costal margin The skin of face and neck showed small, firm, pink infiltrations the size of

a pea, which on histologic examination showed leukemic infiltration. Biopsy of a lymph node also showed the histologic picture of lymphatic leukosis.

The white cell count was 72,000, 85 per cent of which were lymphocytes. In the sternal marrow lymphocytes amounted to 80 per cent of the marrow cells. Treatment with urethane in daily doses of 2 to 4 grams (46 grams in fifteen days) caused a fall of the white cell count from 72,000 to about 5,000 in fifteen days. There was a rise in hemoglobin from 48 to 65 per cent. A further increase to 91-98 per cent occurred later. About one month after the end of urethane treatment 300 mg of folic acid were given in the course of five days. At this time there occurred an increase in white cells to over 10,000, but after 3 grams of urethane daily for fourteen days the leukocyte count returned to normal, where it was maintained six weeks later. At this time the lymph nodes had diminished slightly but were still markedly enlarged. The spleen was palpable only during deep inspiration. The subcutaneous infiltrations were uninfluenced by treatment. The patient felt well, and the temperature was normal.

Estimations of the erythropoietic activity in vitro of sternal marrow were undertaken in both Cases 2 to 3 both before and after treatment. The points of time are indicated in figures 1 and 2, and the results are tabulated in tables 3 and 4.

The calculated erythropoietic activity under various conditions in relation to the average values observed by cross experiments on ten normal marrows and sera were as follows:

	5/31	6/25	10/17	Normal Average
1 $\frac{\text{Path Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0.45	0.82	0.94	0.98
2 $\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0.65	0.96	0.96	0.83
3 $\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0.64	0.87	0.90	0.85
4 $\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Path Marrow} + \text{Path Serum}}$	1.47	1.17	1.02	0.85
5 $\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Path Marrow} + \text{Path Serum}}$	1.44	1.05	0.96	0.86
	6/7 I	8/29 II	10/14 III	Normal Average
1 $\frac{\text{Path Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0.60	0.75	0.80	0.98
2 $\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0.81	0.95	0.96	0.83
3 $\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Norm Marrow} + \text{Norm Serum}}$	0.67	0.70	0.85	0.85

	6/7 I	8/29 II	10/14 III	Normal Average
4 $\frac{\text{Path Marrow} + \text{Norm Serum}}{\text{Path Marrow} + \text{Path Serum}}$	1 38	1 26	1 20	0 85
5 $\frac{\text{Norm Marrow} + \text{Path Serum}}{\text{Path Marrow} + \text{Path Serum}}$	1 12	0 93	1 08	0 86

In both cases, the deviations from the normal values as well as the approach toward the latter after treatment corresponded closely to the findings in pernicious anemia

### SUMMARY

Experiments have been performed on sternal marrow kept alive in vitro with serum as a nutritional medium

Cross experiments with marrow and serum from two normal persons show roughly identical values for erythropoiesis. The same applies to marrows and sera from cases of mild simple anemia.

Marrow from a patient with pernicious anemia showed increased erythropoiesis in vitro if nourished on normal serum, whereas serum from the same case lowered the erythropoiesis of normal marrow. After treatment with liver extract, conditions tended to return to normal.

Marrow from 2 cases of lymphatic leukemia displayed corresponding phenomena, and showed a tendency to return to normal after treatment with urethane and folic acid.

Further investigations along these lines are in progress.

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# STUDIES ON BONE MARROW IN VITRO

## III THE EFFECT OF ANOXIA AND HYPEROXIA ON EXPLANTED BONE MARROW

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THE effect of low oxygen tension on hemopoiesis is well known, since numerous observations have been made on men and animals living at high altitudes. It has been established that the increase in red cells and hemoglobin at high altitudes is not due to hemoconcentration or to an abnormal distribution of the blood, but to a real increase in production. The latter is manifested by a rise in reticulocytes in the peripheral blood and by erythroblastic hyperplasia of the bone marrow. These facts led to the widely accepted conception that even under normal circumstances bone marrow activity is largely regulated by the oxygen tension in this organ. Minot and Castle<sup>1</sup> stated that "in the presence of the necessary chemical factors for normal maturation, the supply of red blood cells is regulated largely by the oxygen tension of the bone marrow" (p. 13). It is, however, unknown whether the bone marrow is directly influenced by the fluctuations of the oxygen tension, or whether the response of the bone marrow is provoked indirectly by other factors, initiated by the low oxygen tension, such as the appearance of incompletely oxidized metabolic products, or vasomotor phenomena. It seemed desirable, therefore, to study the direct effect of various oxygen tensions on bone marrow activity. Isolated bone marrow surviving *in vitro* offered a good opportunity for this purpose.

### MATERIAL AND METHODS

The bone marrow from the tibia of 6-8 weeks old rabbits was used. The technic of explantation was that described in the first paper of this series.<sup>2</sup> The glass tubes containing the bone marrow explants were placed open in a glass flask into which the desired gas mixture was introduced. The gas chamber (diameter 5.5 cm. and height 15 cm.) consisted of two parts, the flask and the cover, connected with a ground glass joint. The flask contained a rack for four culture tubes and a few cc. of water at the bottom to prevent desiccation. After placing the tubes with the cultures in the flask, this was tightly closed with the cover. The cover contained an inlet and an outlet tube of glass, through which the gas was circulated, the inlet tube, reaching the bottom of the flask, the opening of the outlet tube being only a few cm. below the top of the cover. In each experiment two liters of the desired gas mixture were driven through the apparatus and it was then closed by two glass stopcocks in the inlet and outlet tubes.

The gas mixtures were prepared in graduated bottles over water. For mixtures containing less than 20 per cent  $O_2$ , atmospheric air was diluted with nitrogen. For mixtures containing more than 20 per cent oxygen, pure oxygen and nitrogen

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were mixed. Oxygen and nitrogen were taken from commercial cylinders, the nitrogen being passed before use over heated copper turnings. The in-going gas was always at atmospheric pressure. The gas chambers thus prepared as well as the control cultures were placed in the incubator at 37°C for 24 hours. Gas mixtures containing 1, 3, 5, 10, 12, 15 and 50 per cent oxygen were used. The control tubes containing atmospheric air were closed with corks as usual.

After incubation the bone marrow explants were fixed together with the plasma clot in Zenker's fluid. Serial sections 4  $\mu$  in thickness were cut from the material embedded in calloidin-paraffin. They were stained with hematoxylin-eosin and with Giemsa's stain.

### OBSERVATIONS

#### *Cultures of bone marrow incubated in an atmosphere containing 1 per cent oxygen*

The cultures maintained in an atmosphere containing 1 per cent oxygen were severely damaged. There were in all 7 cultures treated in this way and all of them presented a uniform picture of disintegration. The stroma appeared brownish in the hematoxylin-eosin preparation, and the stroma cells were mostly damaged. The hemic cells, erythroid as well as myeloid, showed marked signs of degeneration. The nuclei of the erythroblasts and normoblasts showed margination of chromatin or were fragmented, the promyelocytes and myelocytes showed karyolysis. Nuclear debris was scattered throughout the whole preparation. The only cells well preserved were the megakaryocytes. On the periphery of some explants single well preserved polymorphonuclear leucocytes and their precursors could be observed, and very exceptionally a myelocyte was found in mitotic division.

The surrounding plasma contained undamaged polymorphonuclear leucocytes.

#### *Cultures of bone marrow incubated in an atmosphere containing 3 per cent oxygen*

Eleven explants were incubated in an atmosphere containing 3 per cent oxygen. Practically all of them showed signs of severe injury of the erythroid and the myeloid cells, similar to those seen in the cultures in 1 per cent oxygen. Here also the megakaryocytes were well preserved. The only difference worth mentioning between the cultures in 1 and 3 per cent oxygen was the presence in some explants maintained in 3 per cent oxygen of a peripheral zone containing a greater number of undamaged cells of all varieties and in all stages of maturation. In this zone mitoses were not rare. In the plasma numerous polymorphonuclear leucocytes were present.

#### *Cultures of bone marrow incubated in an atmosphere containing 5 per cent oxygen*

In all 24 cultures were maintained in 5 per cent oxygen. Of these 19 were damaged and the remaining 5 were similar to the controls. The degree of damage was not the same in all injured cultures. Some offered a picture of complete disintegration, others were less uniformly damaged and showed well preserved cells in some peripheral areas of the fragment. In these areas usually the cells of the myeloid series prevailed. The mature leucocytes and erythrocytes were always well preserved.



FIG 1 EXPERIMENT R99 HEMATOXYLIN-EOSIN ( $\times 450$ )

- a) Explant of bone marrow in atmospheric air after 24 hours of incubation
- b) Explant of bone marrow in an atmosphere containing 50% oxygen after 24 hours of incubation

Mitoses were present in the less damaged cultures, but their number was always less than in the controls



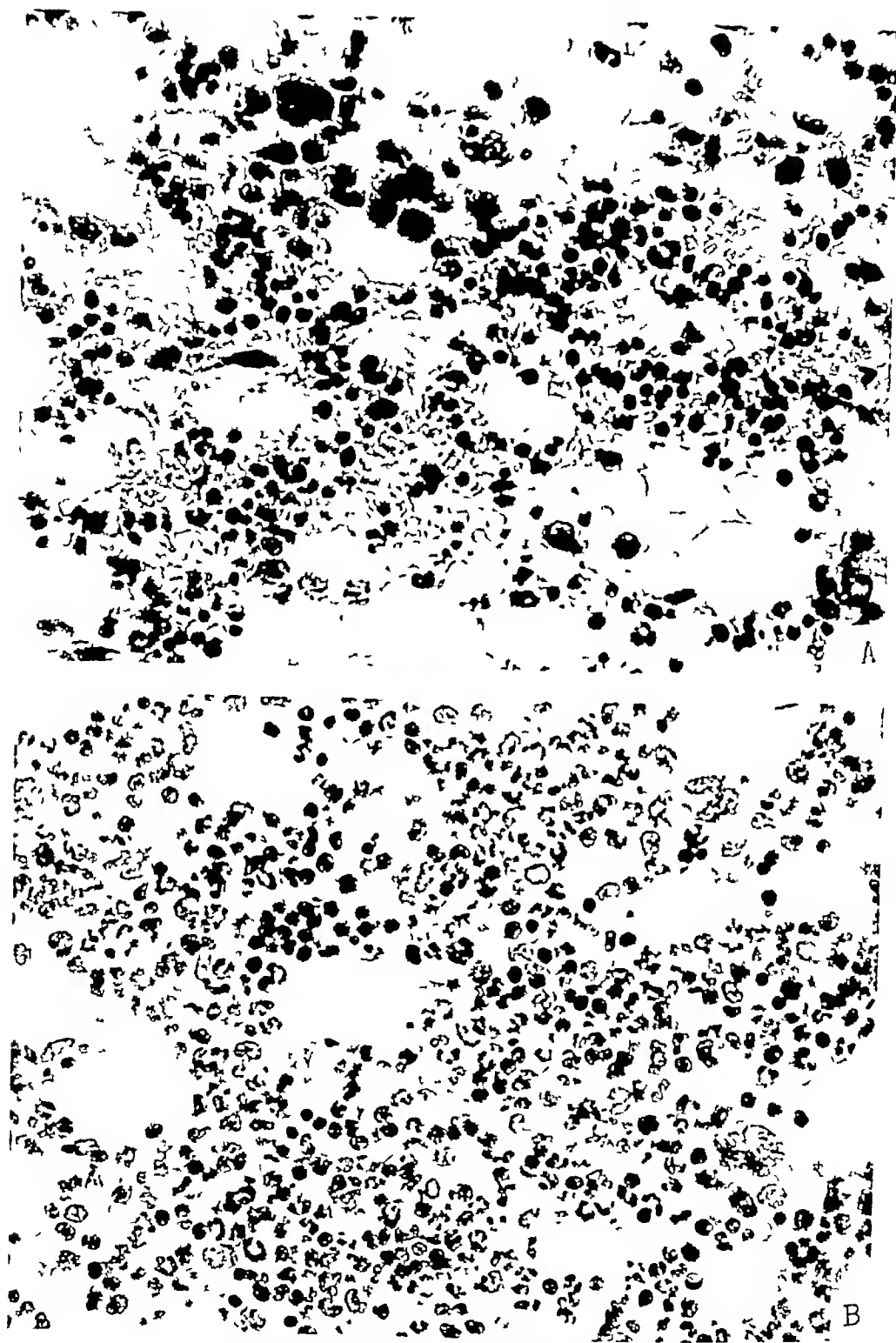


FIG. 2. EXPERIMENT R92. HEMATOXYLIN EOSIN ( $\times 450$ )

a) Explant of bone marrow in atmospheric air after 24 hours of incubation

b) Explant of bone marrow in an atmosphere containing 50% oxygen after 24 hours of incubation

*Cultures of bone marrow incubated in an atmosphere containing 10 and 12 per cent oxygen*

There is no essential difference between the behavior of the bone marrow cultures maintained in an atmosphere of 10 and 12 per cent oxygen, and that of cul-

tures maintained in an atmosphere of 5 per cent oxygen. Out of 28 cultures kept in an atmosphere containing 10 and 12 per cent oxygen, 22 were severely damaged. The remaining 6 were fairly well or well preserved. Here, too, cultures consisting predominantly of erythroid cells were more damaged than those in which the myeloid elements prevailed. The well preserved cultures contained cells in mitotic division, their numbers being, however, less than in the controls.

*Cultures of bone marrow incubated in an atmosphere containing 15 per cent oxygen*

The cultures maintained in an atmosphere containing 15 per cent oxygen presented quite a different picture as compared with the cultures grown in atmospheres with lower oxygen content. Out of 11 cultures 8 were in good condition and similar to the controls. 3 cultures were somewhat damaged, the normoblasts being here also the most affected. Mitoses in the well preserved cultures were nearly as numerous as in the controls.

TABLE 1 — *Effect of Hyperoxia (50% Oxygen) on the Mitotic Activity of Bone Marrow in Vitro*

Animal No	Percentage mitoses	
	24 hours old explants in atmospheric air 'A'	24 hours old explants in an atmosphere containing 50% oxygen 'B'
R92	3 8	7 8
R93	3 8	6 0
R94	1 4	2 4
R95	3 6	4 8
R96	2 8	7 0
R97	3 8	4 8
R98	2 4	4 2
R99	0 6	2 4

*Cultures of bone marrow incubated in an atmosphere containing 50 per cent oxygen*

The cultures maintained in an excess of oxygen presented a picture of definitely stimulated bone marrow. They were characterized by their rich cellularity and excellent condition of the individual cells (figs. 1 and 2). In all, 32 cultures were maintained in an atmosphere containing 50 per cent oxygen. Close examination of the cultures maintained at 50 per cent oxygen revealed a distinct feature in their cellular composition, namely the abundance of mature cells, mainly segmented leucocytes. They contained also a varying amount of precursors, predominantly myelocytes, often in mitotic division. The mitotic activity of the cultures was determined by counting the number of mitoses present in 500 cells capable of undergoing mitotic division. The counts made on the experimental cultures and on the controls revealed that the cultures maintained in 50 per cent oxygen contained a higher number of mitoses than the controls (table 1). It thus seems that the higher  $O_2$ -tension had a stimulating effect on multiplication and maturation.

#### DISCUSSION

No data are available in the literature on the effect of various oxygen tensions on bone marrow cultivated in vitro. The behavior of cultures of other tissues under different oxygen tensions has been the subject of study by a number of investigators.

Burrows<sup>3</sup> (1920-22) working with cultures of heart fibroblasts derived from chicken embryos of 4 to 5 days of incubation, found that they could survive and grow temporarily in the absence of oxygen. Fibroblasts of 10 to 15 day old embryos, however, grew only in an atmosphere containing 1.8 and 5.4 per cent oxygen respectively.

Wind<sup>4</sup> (1926) examined the behavior of Rous sarcoma cultures under entirely anaerobic conditions. He found that in anaerobic conditions the cultures survived and the cells migrated and divided normally for 48 hours. If they were transplanted and cultivated for a further 48 hours under anaerobic conditions, migration occurred, but not as extensively as in the aerobic control cultures. During the third passage the cells in the anaerobic cultures died.

Mottram<sup>5</sup> (1927) studied the effect of reduced oxygen tension on cultures of rat fibroblasts, kidney and Jensen sarcoma. He found that no cell migration took place in the cultures of fibroblasts and kidney at 40 mm Hg (5.2 per cent  $O_2$ ) or lower tensions of oxygen. The cultures of Jensen sarcoma were less sensitive to oxygen want, since cell migration could be observed in these cultures even at 20 mm Hg (2.6 per cent  $O_2$ ). No observations were made as to the occurrence and frequency of cell division in these cultures.

Wright<sup>6</sup> (1928) determined the critical tension required for cell division in cultures of various tissues. He found that the lowest tension at which cell division takes place was for chicken heart fibroblasts about 12 mm Hg (1.6 per cent  $O_2$ ), for Jensen rat sarcoma 6 mm Hg (0.8 per cent  $O_2$ ) and for mouse carcinoma 2146 about 3 mm Hg (0.4 per cent  $O_2$ ). The condition of the cells was good under tensions of 14.7 mm Hg (1.9 per cent  $O_2$ ) and above.

Ephrussi, Chevillard, Mayer and Plantefol<sup>7</sup> (1929), working with cultures of heart fibroblasts of chicken embryos, found that migration took place even in the complete absence of oxygen. However, cell division was inhibited under oxygen tensions of 7 mm Hg (0.9 per cent  $O_2$ ).

Lippmann<sup>8</sup> (1933) observed that outgrowth was possible, but at a very reduced rate, in fibroblast cultures under entirely anaerobic conditions. If respiration was reduced to  $\frac{1}{3}$ , increase of area was diminished to  $\frac{1}{3}$ .

Julius<sup>9</sup> (1933) examined cell cultures in gas mixtures containing oxygen from 0 to 96 per cent. He found increase in area even at 1 per cent oxygen. The optimal conditions for growth were seen in an oxygen concentration corresponding to that of atmospheric air. Mitoses were scarce in pure nitrogen.

Gomirato<sup>10</sup> (1934) found inhibition of growth, but no death of cells, in cultures of heart fibroblasts from chicken embryos maintained in pure nitrogen.

Knake<sup>11</sup> (1934) is the only author who claimed a growth stimulating effect of reduced oxygen tensions (5 per cent) on fibroblast cultures. In very low oxygen tensions, such as 0.2 per cent, as well as in very high oxygen concentrations, i.e. 100 per cent, the cultures were damaged. Oxygen concentrations of 33 and 66 per cent did not affect the growth of fibroblasts.

The investigations cited above indicate that growth of different tissues in vitro is possible under very low oxygen tensions, and even under anaerobic conditions, but that the growth rate is considerably reduced. The few observations on mitoses in tissue cultures under low oxygen tensions always registered inhibition of mitotic activity. According to Wright mitoses in fibroblasts may be present in oxygen concentrations as low as 1.6 per cent, according to Ephrussi et al. even in concentrations of 0.9 per cent. Growth stimulation of mesenchymal cells, measured by the area of outgrowth, caused by low oxygen tensions, was found only by Knake.

Several authors observed an injurious effect of low oxygen tensions on the cells. Wright found injury of fibroblasts in cultures kept in an atmosphere containing only 1.9 per cent oxygen, and Knake in an atmosphere containing 5 per cent oxygen. It must, however, be kept in mind that these authors studied the outgrowing cells and not the cells in the explant itself, as we did. Our experiments on bone marrow growing in vitro show that the reduction of oxygen concentration below 15 per cent has an unfavorable effect on the marrow parenchyma. This is manifested by

the poor preservation of the explants maintained under low oxygen tensions, and by inhibition of multiplication, whereas cell migration took place under all  $O_2$ -tensions tested. The lower the oxygen tensions under which the cultures were kept the more pronounced was the cell damaging effect and the reduction of mitotic activity. Single cells in mitotic division could be found even in gas mixtures containing 1 per cent oxygen, but the mitotic frequency was greatly decreased under reduced oxygen tensions as compared with the control cultures in air. Our observations indicate that erythroid cells are more sensitive to oxygen want than myeloid cells, hence the particularly poor preservation of cultures derived from predominantly erythropoietic bone marrow.

It is generally accepted that the oxygen physically dissolved in the plasma represents the immediate source of supply to the tissues. The quantity of the physically dissolved oxygen depends on the partial pressure of the oxygen in the atmosphere. Thus plasma saturated with alveolar air, containing 14 per cent oxygen, takes up 0.3 per cent oxygen, plasma saturated with pure oxygen takes up 2.2 per cent oxygen. The oxygen consumed by the tissues is constantly replaced *in vivo* by the oxygen dissociated from oxyhemoglobin. In our *in vitro* experiments the only source of oxygen supply is the respective gas mixture in which the cultures are kept. The volume of the gas mixture is comparatively large in relation to the size of the cultures, so that an ample supply of oxygen is secured during the relatively short period of incubation. It must, however, be kept in mind that the oxygen supply may be different in various layers of the culture, the gas penetrating more readily into the superficial than into the deep layers. This reservation holds true for all the cultures, including the controls in air, and our conclusions are based on the comparison of the conditions of the cultures kept in various oxygen tensions with those of the controls.

The lack of bone marrow stimulation by low oxygen pressure cannot be explained by mere technical shortcomings of our method, especially if a comparison is made between the cultures kept under low oxygen tensions and those under high oxygen tensions, i.e. 50 per cent oxygen, when a distinct stimulation could be seen. The stimulation was shown by an increased rate of multiplication as evidenced by the mitotic count, and by accelerated maturation, especially of the myeloid cells. Thus the cultures maintained at 50 per cent oxygen could be easily recognized by the rich cellularity and by the excellent state of the cells. The stimulated maturation, particularly of the granulocytes, caused by an excess of oxygen, is interesting in view of the frequent finding of leucocytosis in the peripheral blood in animals and men under high oxygen tensions<sup>12-14</sup>. It is not, however, yet established whether this leucocytosis is a real one, or whether it is due to inflammatory changes in the lungs caused by inspiration of air rich in oxygen.

It is obvious that observations made on tissue surviving *in vitro* have only a limited value for the interpretation of processes taking place in the living body. This is also true in the case of anoxia and its effects on blood regeneration. However, our findings justify a critical analysis of the widely accepted view that deficient oxygen supply to the bone marrow is the main stimulus for the increased production of red blood cells and hemoglobin.

Paul Bert<sup>14</sup> and Viault<sup>15</sup> were the first to observe increased hemoglobin and red cell formation in men and animals living at high altitudes. Miescher<sup>17</sup> then advocated the view that the physiological regeneration of red cells and hemoglobin was governed by a relative oxygen want existing under normal conditions in the bone marrow. In anoxia bone marrow activity is stimulated to increased blood formation.

Dallwig, Kolls and Loevenhart<sup>18</sup> observed in animals subjected to low oxygen tensions an increase in hemoglobin and a marked extension of the red bone marrow. In accordance with the view of Miescher they came to the conclusion that the bone marrow was stimulated by the low partial pressure of oxygen. The same view was adopted by Schaumann and Rosenquist<sup>19</sup> and by Loewy.<sup>20</sup>

There are certain facts, however, already pointed out by Miescher,<sup>17</sup> Morawitz<sup>21</sup> and by Loewy<sup>20</sup> which are difficult to fit in with the theory that reduced oxygen tension in the bone marrow itself is responsible for the physiological and the accelerated blood regeneration. If oxygen want is directly responsible for the increased hemopoiesis at high altitudes, one might expect a certain relation between the diminution of the oxygen tension and the degree of blood regeneration. This is, however, not always the case, definite bone marrow stimulation is already found at altitudes of 600-700 meters where a diminished oxygen tension in the tissues can hardly be expected. The increased blood production at altitudes of 2000 to 5000 meters can easily be explained by the reduced oxygen tension, but this increase does not continue at higher altitudes, such as 6000 meters and more, where the proportion of reduced to oxygenated hemoglobin increases rapidly (Hurtado<sup>22</sup>).

According to the theory of the stimulating effect of oxygen want, one should assume that strenuous exercise which increases the anoxia, should also augment the blood regeneration. This, however, is not in agreement with the observations of Cohnheim and Kreglinger<sup>23</sup> and Gross and Kestner<sup>24</sup> who found that strenuous exercise at an altitude of 10,000 feet produced a fall in hemoglobin.

Furthermore, if oxygen want is the factor responsible for bone marrow stimulation, one should expect that atmospheres rich in oxygen should depress bone marrow activity. Experiments to this effect made on animals reveal contradictory results. Some authors found a decrease,<sup>25a, 25b</sup> others an increase in red cells.<sup>12, 26-28</sup> According to Karsner,<sup>29</sup> prolonged exposure to high oxygen concentrations produced no changes in the erythrocyte count. In this connection the experiments of Boycott and Oakley<sup>30</sup> are significant. These authors failed to find in rats an inhibiting effect of high oxygen concentrations on the regeneration of red blood cells after hemorrhages. The reticulocyte response was rather greater in the rats exposed to 65 per cent oxygen after bleeding and the color index was regularly lower than in the controls.

The definite stimulation of bone marrow activity at high altitudes may be due not to oxygen want in the marrow, but to compensatory factors initiated by the low oxygen tension of the atmosphere. The factors which come into play under low oxygen tension and which may compensate for the oxygen want are increased respiration, increased arterial pressure, increased cardiac rate, and at very high altitudes, increased minute cardiac output. Sands and de Graaf<sup>31</sup> found in dogs subjected to a reduced oxygen tension in the inspired air an increased systolic discharge, an increased heart rate and a reduced peripheral resistance, all factors leading to an increase of the minute flow of blood through the body. Thus it may

be assumed that in moderate degrees of anoxemia the blood flow may also increase in the bone marrow so that actually there may be no oxygen want, but even a compensating hyperoxemia in this organ

According to Gesell<sup>32</sup> a greater utilization of oxygen by the tissues and an actual increase in oxidative energy occurs even during the period of oxygen want, when a normal increase in ventilation is allowed to combat the shortage of oxygen in conjunction with circulatory adjustments. Thus one could assume that a state of "anoxic hyperoxia" might arise in analogy to the "hyperoxic anoxia" postulated by Bean<sup>33</sup> in oxygen poisoning

The effect on the bone marrow of compensatory processes taking place in anoxic conditions might explain the increased blood regeneration already found in low altitudes, and the fact that at very high altitudes, where the circulatory and respiratory adjustments may become insufficient, no further increase in blood formation occurs

### SUMMARY

The effect of various oxygen tensions on explanted bone marrow fragments was studied. It was found that gas mixtures containing 1, 3, 5, 10 and 12 per cent oxygen have an injurious effect on hemic cells. Bone marrow maintained in these gas mixtures showed various degrees of degeneration, which was the more pronounced the lower the oxygen tension. Mitotic activity was also found to be reduced under the influence of low oxygen tension.

Bone marrow cultures maintained in a gas mixture containing 15 per cent oxygen did not show appreciable changes and were similar to the controls.

Increased rate of maturation and multiplication occurred in bone marrow cultures maintained in an excess of oxygen, i.e. 50 per cent.

The significance of these findings in the light of observations on the effect of anoxia *in vivo* has been discussed, and reported findings on the effect of low oxygen tensions on other tissues *in vitro* have been briefly reviewed.

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## ELECTRON MICROSCOPE STUDIES OF BLOOD CELLS

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IN AN earlier report<sup>1</sup> we have described briefly the technics utilized in the application of the electron microscope to the study of the blood cells and the preliminary results obtained by these technics. They consisted of the study of lymphocytes, macrophages and neutrophilic leukocytes of experimental inflammatory exudates of man as well as the neutrophils, neutrophilic metamyelocytes, lymphocytes and their precursors, red corpuscles and the later stages of normoblasts obtained from the hematopoietic organs of man.

Several excellent, electron micrograph studies of other tissue cells have already been reported, even though the study of these relatively large, thick and more delicate tissue cells with the electron microscope has imposed somewhat greater problems than has the study of bacteria and viruses. Scott and Packer<sup>2-4</sup> reported on the localization of minerals, particularly calcium and magnesium, in striated and smooth muscle,<sup>5</sup> and Scott and Anderson<sup>6</sup> studied connective tissue. Schmitt, Hall, and Jakus<sup>7-13</sup> have contributed much to our understanding of protoplasmic fibrils in their studies of collagen fibers, protozoan trichocysts, muscle fibers, sperm, flagella, cilia and fibrin. Richards, Anderson and Hance<sup>14</sup> depicted electron micrographs of striated muscle. Clark, Barnes and Baylor<sup>15</sup> and later Buchholz<sup>16</sup> have reported on chromosome structure. Claude and Fullam<sup>17-18</sup> studied isolated mitochondria and sections of guinea pig liver with the electron microscope. Porter, Claude and Fullam<sup>19</sup> devised a method of electron microscopy of tissue cultures of fibroblasts and nerve endings.

A small number of papers have dealt with electron microscope studies of the blood cells or their derivatives. To the best of our knowledge, save for our preliminary report mentioned above, they have concerned themselves with studies of the blood platelets or the mature red blood corpuscles. Wolpers and Ruska<sup>20</sup> reported on the structure of the blood platelets and their relation to fibrin. The platelet granulomere was found to consist of from 60 to 120 granules, rarely as few as 20 granules were seen. The hyalomeric protoplasm exhibited a fine framework-like structure (their figure 9). In the course of the clotting process their electron micrographs depict the platelets swelling, aggregation of granules, vacuole and process formation by the hyalomere, loss of all save a remnant of hyalomere, but finally, persistence of the granules as a place of deposition or attachment for the fibrin micelles. In a later paper Ruska and Wolpers<sup>21</sup> detected regularly spaced, dark cross bands in fibrin fibrils. Wolpers<sup>22</sup> separately investigated the structure of the red corpuscular membranes. Electron micrographs of red blood corpuscles and hemolysed erythrocytes are depicted by him. The membranes of the latter were studied after lipid extraction and were composed of an intricate meshwork of long and slender protein fibrils. Barnes, Burton and Scott<sup>23</sup> portrayed in their Fig. 5 a polystyrene-silica replica of red blood corpuscles. Jones<sup>24</sup> recently

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was able to make methacrylate surface replicas obtaining micrographs of blood cells of a quality similar to those of polystyrene

### MATERIALS AND METHODS

We have employed several technics. First, lymphocytes, macrophages and neutrophilic leukocytes of the inflammatory exudate of man were obtained by applying formvar-covered glass cover slips over small lesions produced in the corium of the forearm of human volunteers. To produce these small lesions, the forearm was first cleansed with alcohol, then by means of a sterile scalpel or sterile razor blade, the epithelium was slowly scraped away over a circumscribed area 2 to 3 mm in diameter, until the papillary layer of the corium was exposed. A few minute bleeding points indicate the proper depth. Care must be taken to avoid lacerations or obvious hemorrhage into the bed of the tiny lesion. In keeping with Kolouch's<sup>25</sup> investigation of lymphocytic functions in rabbits with the light microscope, a small drop of egg white was next placed on the lesion, to increase the lymphocytic content of the cellular exudate.

A formvar-covered glass cover slip was next placed over the lesion, film side down. The cover slip was then covered with a square of cardboard and the entire preparation was covered with surgical adhesive tape, approximately 2 by 4 inches in size. Additional pressure over the lesion can be obtained by placing a flat-bottomed cork over the adhesive surmounting the cover-slip area, the cork is then held in place by a long narrow adhesive band.

In an hour or two the cells of the inflammatory exudate migrate to the under surface of the formvar film of the cover-slip, spreading themselves out in a thin, single-celled layer. When at definite timed intervals, it is desired to sample the cellular exudate, the film-covered cover-slip is removed from the lesion and the lesion is immediately recovered by a second film-covered glass cover-slip. The cells which migrated to the undersurface of the film-covered cover-slip, while still on the cover-slip, are immediately quick-frozen and dehydrated *in vacuo* according to Wyckoff's<sup>26</sup> modification of the Altmann-Gersh<sup>27, 28</sup> technic. It should be noted that earlier, Scott and Packer had prepared striated muscle for electron microscopy by plunging the muscle into chilled isopentane before dehydration.

The final specimen mounting is performed as follows. Place the specimen screen over a suitable cell-containing area which has been selected for observation in the electron microscope. The suitability of such areas first may be adjusted roughly by examination under the optical microscope. Next a strip of Scotch tape is affixed over the specimen screen as well as over the entire slide. The Scotch tape is then removed and the film adheres to it. Inasmuch as the specimen screen is interposed between a small portion of the film and the Scotch tape, removal of the specimen screen at this stage will carry along an equal area of the film. The intact cells are thus actually mounted and observed between the specimen screen and the film. This is a direct mounting technic, then, and these specimens are not replicas.

Another satisfactory method of sampling the fresh exudative cells of man was obtained by imbedding a formvar-covered 200 mesh specimen screen, film side down in the lesion instead of surmounting the lesion with the formvar-covered

glass cover-slip The screen is covered as before, the sterile glass cover-slip is also included, this time serving merely as a bland protective surface about the screen For a few hours the diapedesis and extravasations of red corpuscles into the lesion, as well as the cellular debris, will obscure the screens for electron micrographic purposes But if the film-covered screens in the lesion are replaced by means of fine sterile forceps at intervals of several hours, the exudate will gradually thin out and satisfactory preparations can be obtained These formvar-covered specimen screens were prepared in the usual manner as outlined in current texts of electron microscopy

A third technic employed for the study of the blood cells of the lymph nodes, spleen and bone marrow of man consisted of obtaining imprint preparations of portions of such organs upon formvar-covered glass slides Both fresh surgical pathology specimens and specimens obtained at autopsy were utilized The freshly cut surface of a small portion of the organ is very gently brought in contact with a formvar-covered glass slide, without any smearing or pressure The preparations so obtained were also frozen and dehydrated in vacuo The films were then transferred to screens by the modified stripping technic described above in which the cells were again retained intact Rarely in clinical work when more elaborate apparatus was not available, the imprint preparations were merely air-dried

Downey and his associates in this country<sup>29-31</sup> have been persistent advocates of the imprint technic as an adjunct to the detailed cytologic examination of the blood cells with the optical microscope Sweitzer and Winer<sup>32</sup> have recently published an illustrated description of this technic for optical examination Once experience has been gained in optical studies by means of the imprint technic, its application to electronic studies is not difficult

## RESULTS

Fig 1 is an electron micrograph of a mature red blood corpuscle ( $\times 5400$ ) It is shown merely for purposes of size orientation A micron scale is also shown Such a corpuscle shows no stromal details, but Wolpers<sup>22</sup> work on the membranes of hemolysed corpuscles has shown that the surface of the membrane is composed of a fibrous network following the surface curvature Fig 2 ( $\times 5400$ ) is an electron micrograph of an orthochromatic normoblast from a bone marrow imprint The distinct, fine chromatin particles, are partially obscured by denser, coarse chromatin clumps as the red cell undergoes its natural pyknosis The central cell in the next electron micrograph, fig 3, corresponds to the polychromatophilic stage of normoblastic development, this cell was magnified to  $\times 5400$  Note the larger areas of finely dispersed chromatin in this nucleus, indicating less nuclear maturity The cytoplasm of both these cells (fig 2 and 3) is scant and not remarkable at this magnification

Fig 4 is an electron micrograph of a small macrophage or histiocyte, obtained from the inflammatory exudate of the arm, magnified to  $\times 5400$  This cell measures 9 by 15 microns It was obtained 16 5 hours after inflammation had been produced The horseshoe shaped nucleus, the nuclear-cytoplasmic interface, the cytoplasm and the cytoplasmic surface are plainly seen A portion of the same cell magnified

to  $\times 18,000$  in fig 5 shows an interlacing network which goes to form a type of cellular skeleton. The greater portion of fig 5 depicts the delicacy of the nuclear framework. Only the lower central portion of the micrograph contains a small area of the cytoplasmic bay, but it is sufficient to afford ample study of its con-



FIGS 1 TO 4

trasting structure. The nuclear interstices, are, on the average, smaller than those within the cytoplasmic protoplasm, although some of the smaller cytoplasmic spaces measure only  $15 \times 30$  millimicrons and some of the larger nuclear spaces measure  $120 \times 200$  millimicrons. The nuclear strands or fibrils appear to be thicker than the corresponding structures in the cytoplasmic protoplasm.

Fig 6 ( $\times 21,000$ ) is an electron micrograph of this cell depicting the major portion of the nuclear indentation and cytoplasmic bay areas. The U-shaped portion of nuclear membrane can be observed as an anchoring membrane for both



FIGS 5 AND 6

cytoplasmic and nuclear structural bands. Fig 7 ( $\times 94,000$ ), is again a micrograph of a portion of this same cell. The upper portion of this figure is cytoplasm, the lower portion nuclear protoplasm. The nuclear membrane runs obliquely across



FIG 7

the lower third of the figure. The individual fibrillar cross-pieces, which can be made out easily in either the nuclear or cytoplasmic protoplasm, actually measure

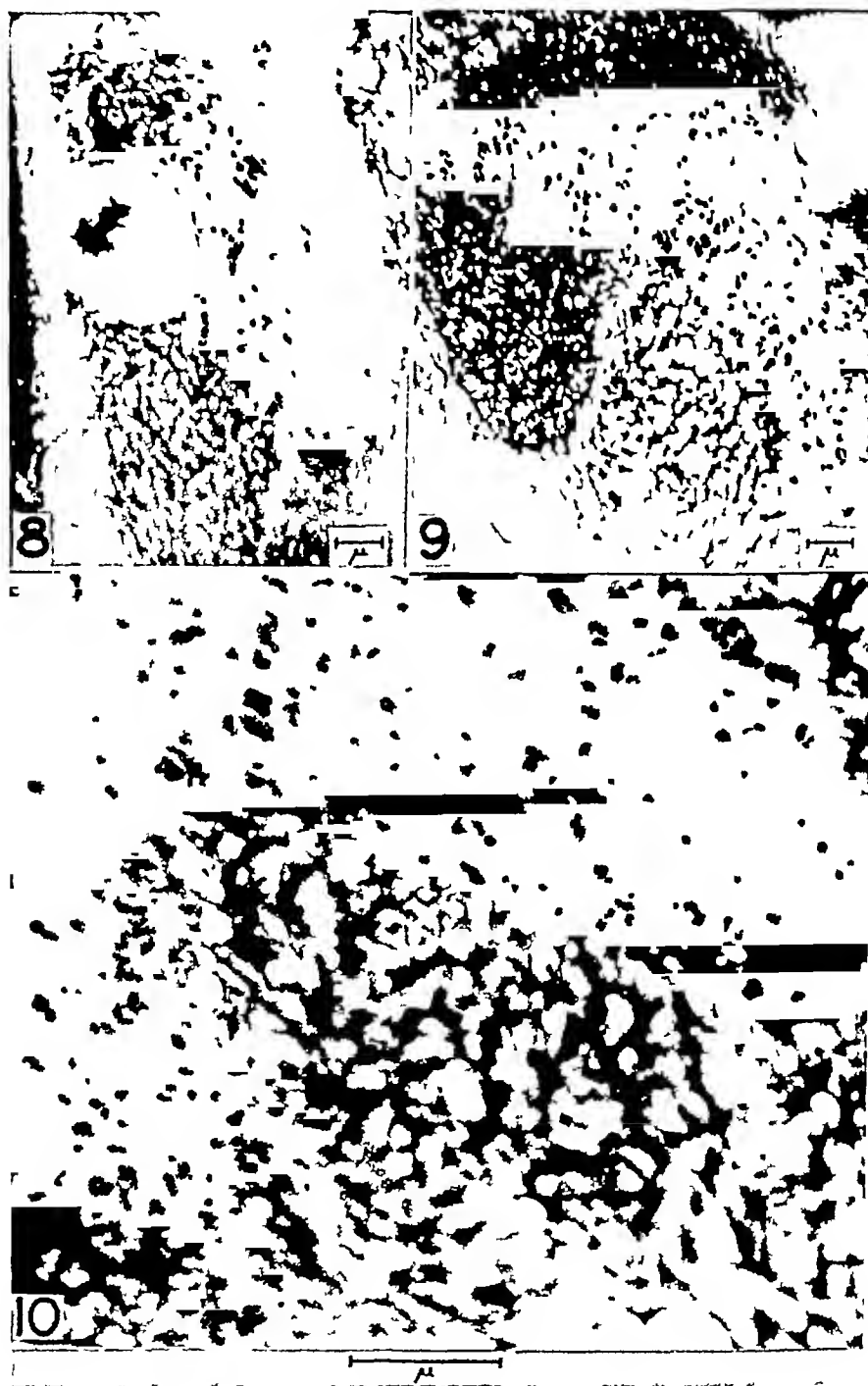
only 140 to 200 or so Angstrom units in breadth and about 1000 Angstrom units in length

Fig 8 is a micrograph of a second macrophage from the 16 5 hour stage of inflammation in man To the best of our knowledge this is the first electron micrograph depicting phagocytosis Cellular debris has been ingested by a small mononuclear This phagocyte only measures  $7 \times 10$  microns A delicate rim of cytoplasm is depicted bridging over the particle The vacuolar lining wall is formed by loops of cytoplasmic fibrillae which usually arch out towards the vacuolar lumen and return into the cytoplasm after forming a scallop-like lining for the vacuole Occasionally free ends of these fibrillae appear to project for short distances into the vacuole

Fig 9 ( $\times 5400$ ) is a micrograph of a third macrophage from the eleventh hour of inflammation in another lesion of the forearm Fig 10 ( $\times 18,000$ ) is a high power view of a portion of the nucleus and cytoplasm of the same cell The structural features of the horseshoe shaped nucleus, cytoplasm and nuclear membrane can also be observed in this cell In all of these macrophages at the site of the cytoplasmic surface, the fibrillar structures of the protoplasm seem to project quite freely, without forming a distinct membrane This could be due to slight shrinkage of the cytoplasm with the initial impact of the electrons, if not, the well known sticky character of the surface of such cells may be in some way related to this finding

Fig 11 ( $\times 11,000$ ) is a micrograph of the peripheral portion of the cell body of a fourth macrophage taken from the same lesion as the previous cell at the eleventh hour of inflammation Several linear arrangements of feather-like, clear areas radiate throughout the cytoplasm This finding warrants some discussion of ice crystal artefacts, although, for reasons which will appear below, we do not feel that such spaces represent ice crystal artefacts Simpson<sup>33</sup> analyzed the various factors influencing ice crystal artefacts produced in protoplasm by the Altmann technic Although his work was performed with the light microscope, he found that such artefacts were small enough to disrupt nuclear detail and produce fine reticulation of the cytoplasm in sections of guinea pig liver cells at some distance from the surface of the tissue block The operation of such factors in the production of the extremely small interstitial nuclear and cytoplasmic spaces of our figs 4 to 11 cannot, of course, be definitely excluded at this time Examination of Simpson's fig 3, plate I, reveals, however, that his ice crystal artefacts are many hundreds of times larger than the protoplasmic spaces of our macrophages Kistler's<sup>34</sup> concept must also be considered, namely that the water of biological tissues is in the form of small isolated droplets and may undercool without freezing It is probable that the water content of the macrophages is higher than that of the blood cells themselves Certainly it is true that according to Simpson's standards, our preparations approach the optimum as to size of the piece of tissue frozen and ratio of large surface area to small tissue volume It is unlikely that an insulating gaseous phase of any consequence enveloped the cells of our preparations under the conditions of the technic employed Wolpers and Ruska<sup>20</sup> demonstrated a type of protoplasmic framework similar to that under discussion, in the hyalomere of blood platelets Their electron micrographs are of platelets not submitted to

freezing of any type Wolpers'<sup>22</sup> electron micrograph of an unfrozen red corpuscular membrane likewise shows a type of delicate fibrillar structural framework, dem-



FIGS 8 TO 10

onstrating that the fibrillar structure of protoplasm need not necessarily be a product of ice crystal artefact



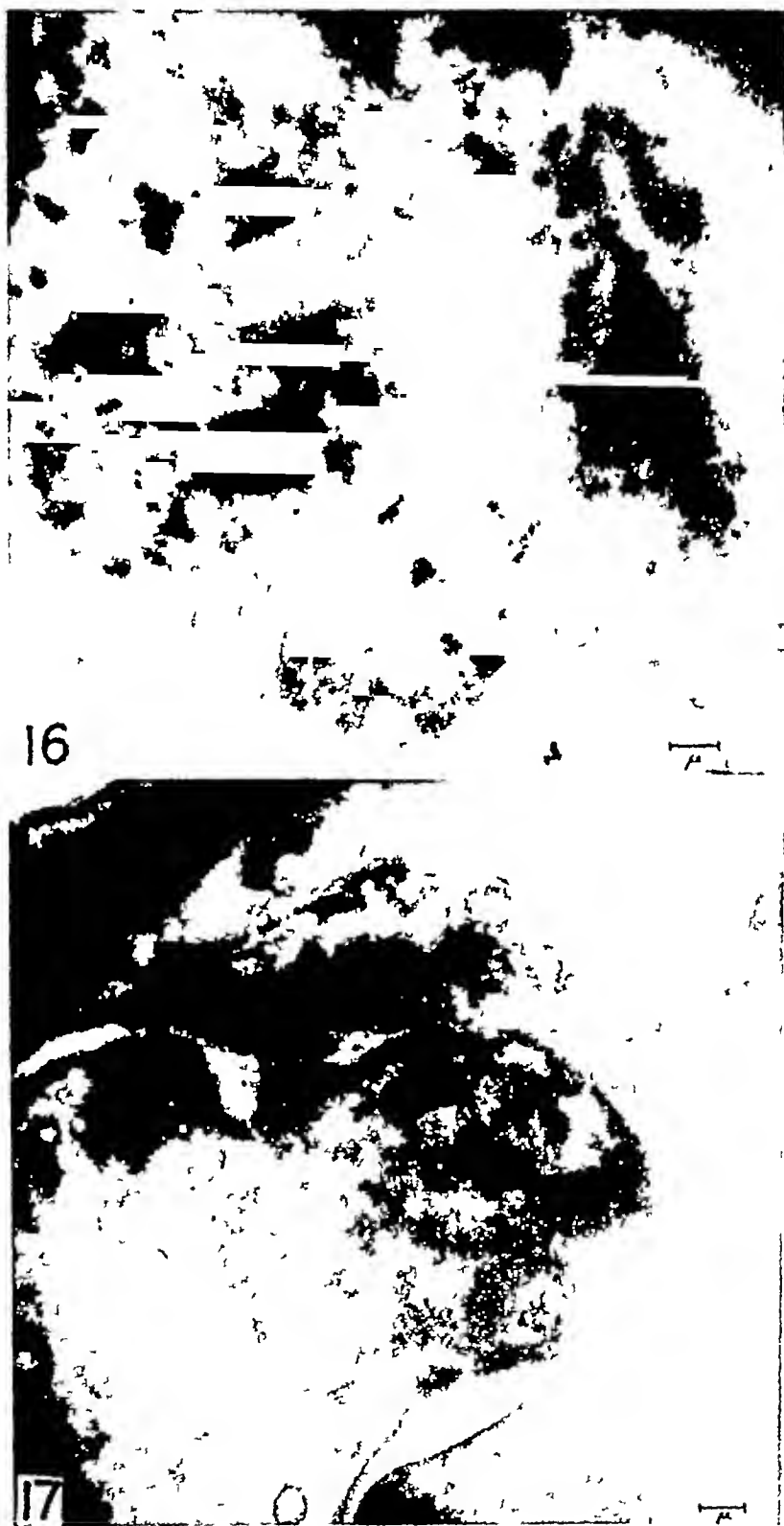
FIGS 11 AND 12

Chambers and Hale,<sup>35</sup> on the other hand, employed internal freezing to throw light on structural elements within the cell instead of looking upon it as a possible source of ice crystal artefacts. They demonstrated that the advance of ice columns





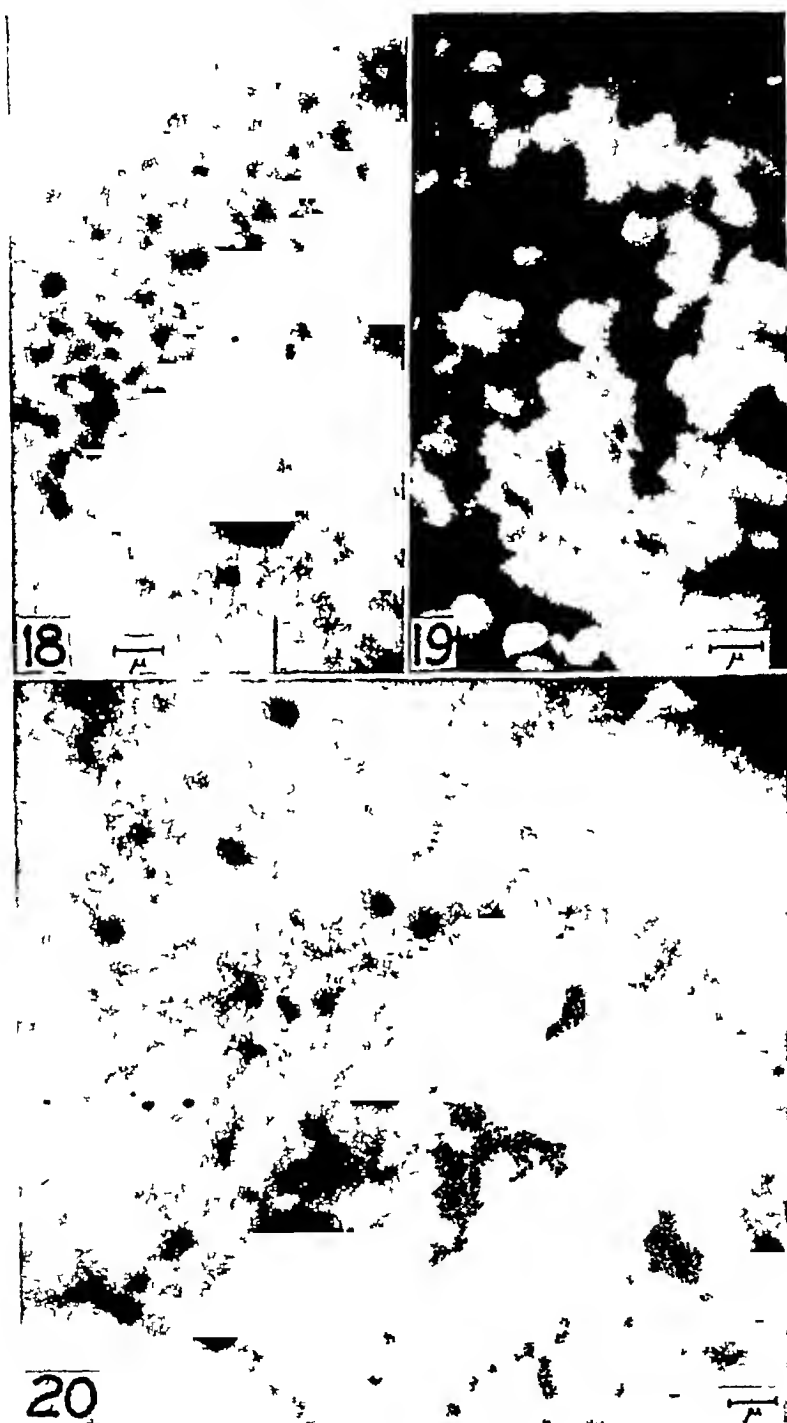
FIGS. 13 TO 15



FIGS 16 AND 17

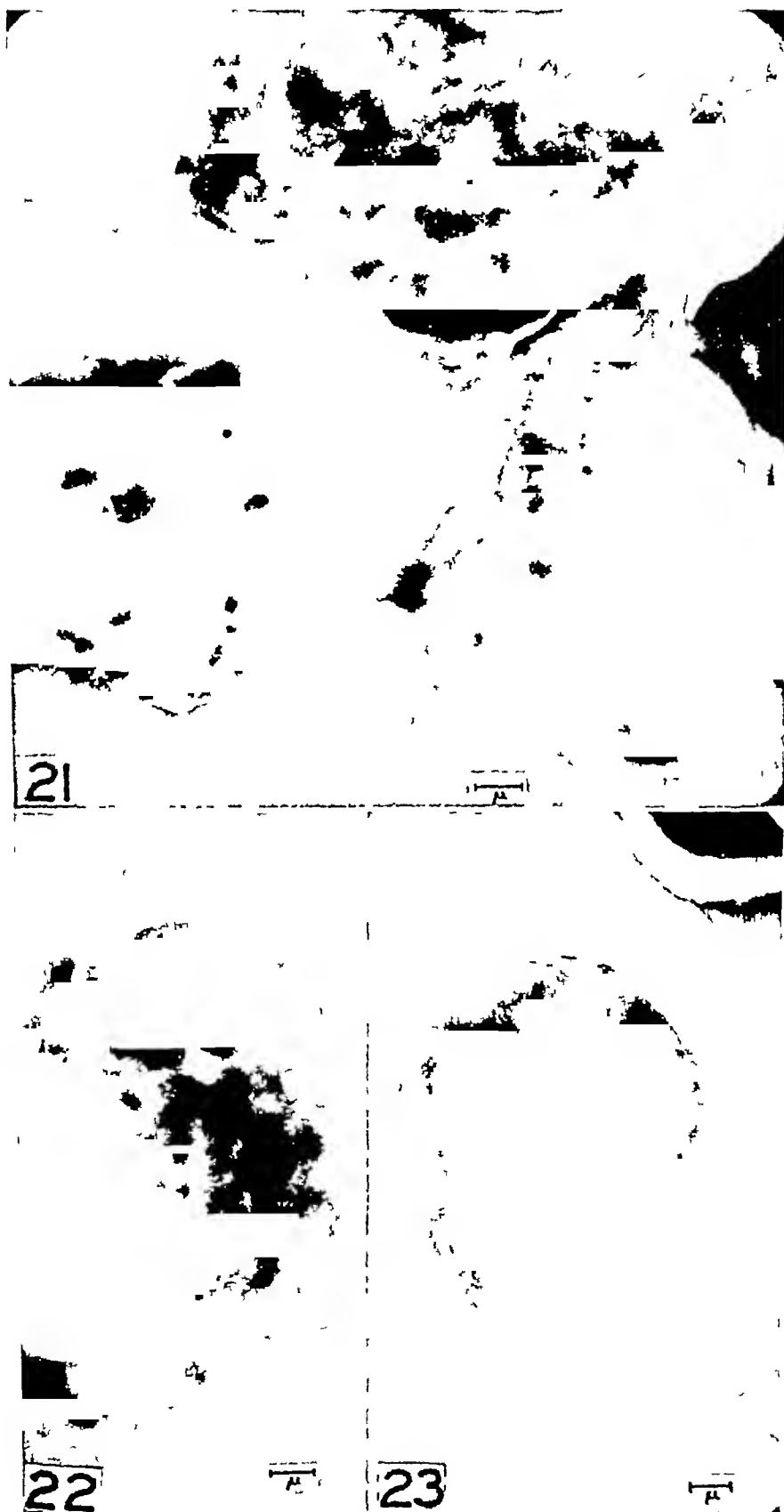
within the protoplasm of frog muscle cells occurred by congealing of the water present in advance of the crystal. Hence it is reasonable to suppose that the steady and uniform advance of the longitudinal ice columns during the internal

freezing of a muscle fibre is along uninterrupted fluid interstices between more solid constituents " To them, the random spreading of fine feathery crystals within the



FIGS 18 TO 20

protoplasm of amoebae indicated a lack of a complicated framework structure. Because of temperature differences between the interior of the cell and its environ-



FIGS 21 TO 23

ment, they assumed the existence of "still finer capillary spaces within the cell." The finding of free fibrillae at the cytoplasmic surface of our macrophages is open to the criticism of their view that to attain internal freezing, the plasma membrane breaks down.

Fig. 12 ( $\times 5400$ ) is a micrograph of one neutrophilic leukocyte and a portion of two others. Neutrophil granules, seen only indistinctly heretofore with the optical microscope, when seen at  $\times 18,000$  (fig. 13) are round, oval, or rod-shaped structures. At  $\times 47,000$  (fig. 14) and  $\times 72,000$  (fig. 15) the neutrophil granules are clearly demarcated. They measure from 70 by 85 millimicrons up to as much as 465 by 650 millimicrons. Figs. 13 and 15 are from the same frozen and dehydrated preparation. Fig. 14 was taken from a preparation which was merely air-dried.

Fig. 16 is an electron micrograph ( $\times 5800$ ) of a neutrophilic metamyelocyte or juvenile from the bone marrow. Its deeply indented nucleus and granular cytoplasm are characteristic features. Fig. 17 is a micrograph ( $\times 5400$ ) of a neutrophilic myelocyte with its round nucleus and the less abundant granular content of its cytoplasm. Fig. 18 ( $\times 5400$ ) is a portion of another neutrophilic myelocyte. Fig. 19 ( $\times 5800$ ) depicts a portion of the nucleus (to the left center) and cell body of an eosinophilic leukocyte. The eosinophil granules are nearly spheres and are quite clear and can be best made out as they overlie the nucleus. Eosinophil granules range from  $780 \times 830$  up to  $1050 \times 1150$  millimicrons in size. Fig. 20 ( $\times 5800$ ) represents one of the granulocyte precursors at the promyelocyte or possibly the leukoblast stage. The granules in the cytoplasm are less well-defined, and it is impossible to state whether they represent specific or azurophil granulation. The cytoplasm is more abundant than the preceding stages. The nuclear membrane is exceedingly thin, the chromatin pattern is much finer than the older developmental forms and three or four nucleoli are in evidence. All the developing granulocytes were obtained from bone marrow preparations.

Fig. 21 ( $\times 5400$ ) is a micrograph of a group of three normal, small and medium-sized lymphocytes from a lymph node. This preparation was not frozen or dehydrated, it was merely air-dried. All other preparations with the exception of Fig. 14 were quick-frozen and dehydrated. The coarse chromatin pattern and scant cytoplasm are well shown. Fig. 22 ( $\times 5400$ ) is a micrograph of an extremely immature lymphocyte. The fine chromatin pattern and distinct nucleoli are in sharp contrast to the cells in fig. 21. The immature lymphocyte of fig. 22 was taken from the spleen of a patient with lymphatic leukemia. Fig. 23 ( $\times 5400$ ) depicts a leukemic lymphocyte in amitosis, from a lymph node.

## DISCUSSION

Newer concepts concerning the finer structure of protoplasm are just beginning to find their application to the study of the white blood cells. De Bruyn<sup>36</sup> has recently interpreted the amoeboid movement of leukocytes in the light of Frey-Wysslings<sup>37</sup> structural schemata for protoplasm. The modern concepts<sup>37-39</sup> of protoplasmic structure comprise molecular configuration of polypeptide chains with their associated intermolecular forces. Between such molecular protein structures are water, salts, mobile protein, and lipid, as well as submicroscopic

particulates which serve in the mediation of special chemical processes. The structural units themselves include at least fibrous and nucleoproteins as well as lipids. The intermolecular forces of structural proteins result in part in their being joined at certain points by chemical bonds. De Bruyn<sup>36</sup> envisions contraction of leukocytic protoplasm as being due to increased binding of such molecular structures at more and more points by chemical bonds, thus narrowing "the meshes of this three-dimensional reticulum," or in keeping with Astbury's<sup>39</sup> findings, as due to actual folding of the polypeptide chains. Solation, then, would involve the loosening of many, but not all, bonds with a resultant loosening of the structural mesh-work, whereas cytoplasmic gelation would mean the locking of more side chains with the structural proteins approaching the formation of a three-dimensional reticulum.

Wolpers'<sup>22</sup> micrograph (his fig. 7) of the structure of the nonmotile red blood corpuscle depicts a tightly knit framework suggesting that this may be composed of a mesh-work of long and narrow protein fibrils. The fibrillar structures of platelet hyalomere are not so closely bound as an examination of Wolpers and Ruska's<sup>20</sup> micrographs of such structures reveals. We would expect less definite orientation of the structural proteins of the more motile leukocytes and definite orientation in the depolarized derivatives. Menkin<sup>40</sup> has commented on the fact that macrophages are much more resistant to deformation than polymorphonuclear leukocytes and cites as evidence the fact that rabbit macrophages resist stresses at water-oil interfaces which severely damage polymorphonuclear leukocytes.

The macrophage stage of the various hematogenous and histogenous cellular sources of macrophages is likewise apparently marked by a tendency to sluggish pseudopodial activity. Our micrographs (figs. 4 to 11) of macrophages reveal a complex fibrillar mesh-work with definite structural orientation of both cytoplasm and nucleus. These findings are at least in keeping with modern functional and structural concepts of protoplasm and are suggestive that some of the structural units of protoplasm can be oriented at times to form a framework of linear molecules when the situation requires such reorientation.

#### SUMMARY

Several technics were employed for the application of the electron microscope to the study of the blood cells. First, lymphocytes, macrophages, and neutrophilic leukocytes of acute inflammatory lesions in man were prepared by imbedding formvar-covered screens in the corium of the forearm. Exudative cells which migrated to the undersurfaces of the specimen screens were quick-frozen and dehydrated in vacuo, according to a modified Altmann-Gersh technic. In some cases formvar-covered glass cover slips were substituted for the screens in the lesions, in which case an extra step was needed to transfer the preparations to specimen screens.

In a further technic, blood cells of lymph nodes, spleen and bone marrow of man were imprinted upon glass slides covered with formvar. These preparations were also frozen and dehydrated in vacuo. The films were then transferred to screens by a direct method in which the cells were retained intact.

Neutrophil granules, seen only as irregular, indistinct granules with the optical

microscope, are actually round, oval, or rod-shaped structures measuring from 70 by 85 millimicrons up to as much as 465 by 650 millimicrons

Immature blood cell precursors were found to possess fine chromatin patterns with distinct chromatin-parachromatin distinction and well-demarcated nucleoli

At the higher magnifications made possible by electron microscopy, a new level of cytostructural organization appeared in the mononuclear phagocytes or macrophages. Individual fibrillar structural units can be made out in both the nuclear and cytoplasmic protoplasm which can be measured in Angstrom units. The nuclear protoplasmic interstices so formed are smaller than the corresponding cytoplasmic areas. The nuclear membrane is actually an anchoring structure for both nuclear and cytoplasmic fibrillar cross-pieces. Phagocytosis is depicted and the wall of the phagocytic vacuole is described. Finally, the observed cyto-structural features of the blood cells are discussed in the light of newer concepts of the finer structure of protoplasm.

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# CASE REPORT

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## APLASTIC ANEMIA

### SECONDARY TO GOLD THERAPY

By WILLIAM J. FITZPATRICK, M.D., AND STEVEN O. SCHWARTZ, M.D.

GOLD has become widely used as a therapeutic agent.<sup>3,7</sup> Its greatest accepted field of effectiveness is in the treatment of rheumatoid arthritis. It has also been employed therapeutically for lupus erythematosus and, for many years, especially in France, for all types of tuberculosis. Toxic effects are frequent in large series of gold treated cases. Hartfall et al.<sup>7</sup> reported 41.9 per cent incidence of toxic reactions in the treatment of 900 cases. Of these reactions 6 per cent were severe, with 7 deaths: 3 from hemorrhagic purpura, 1 from agranulocytosis, 2 from liver necrosis, and 1 from exfoliative dermatitis. There were adverse hematologic reactions in 10 cases in all. In 1942 Cecil et al.<sup>3</sup> reported on 245 cases treated with gold. Of these, 105 had toxic manifestations (42 per cent). 4 cases were hematologic, and included 3 hemorrhagic purpuras and 1 agranulocytosis. A recent report by Lockie et al.<sup>9</sup> mentions toxic reactions in 20 per cent. Whether these toxic manifestations are due directly to the drug or to individual sensitivity is as yet unknown. Clinical evidence favors individual sensitivity, since there is often little correlation between the amount administered and the severity of adverse effects. Experimental evidence also favors hypersensitivity,<sup>8</sup> since toxic symptoms, at least of the hematologic type, could not consistently be reproduced in animals. Hematologic manifestations of toxicity, though relatively rare, are among the most serious. Of these, thrombocytopenia is the most common with agranulocytosis next in frequency and aplastic anemia the rarest and most serious.

Dameshek<sup>6</sup> reviewed the literature in 1934, and found 6 cases of aplastic anemia due to gold therapy. To these he added a case. In 1939 Wintrobe et al.<sup>13</sup> collected 6 more cases and added still another. Four more cases have appeared in the literature since that time. Giraud<sup>6</sup> mentioned a case presenting 750,000 red blood cells, 1,800 white blood cells and a granulopenia of 30 per cent following gold therapy. Pergola<sup>10</sup> reported the case of a 35 year old dancer who developed aplastic anemia following her second course of gold in which a total of 1 Gm. of gold salt was given. This case had the classic triad of anemia, granulopenia, and thrombopenia, and in addition a fatty marrow, 80 per cent of whose cells were lymphocytes. She recovered after a period of 8 months. Armas Cruz and coworkers<sup>1</sup> described a case of aplastic anemia which occurred after a total of only 3 ampules of "Salganol" in-

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intramuscularly and 2 injections of 0.25 and 0.50 gr of gold chloride intravenously. Sternal marrow revealed marked fibrosis. The patient was being treated for lupus erythematosus. Aubert<sup>2</sup> in 1946 published the case report of a 36 year old female who received a total of 2.1 Gm of "Salganol" and who, 5 months later, developed classic signs and symptoms of aplastic anemia.

Altogether 18 cases of aplastic anemia following gold therapy have been reported, with 4 recoveries. We are describing 2 more cases, both terminating fatally, bringing the total to 20. The cases reported fulfill the diagnostic criteria of gold therapy followed by a syndrome of anemia, leucopenia, granulopenia, thrombocytopenia and hypoplasia or aplasia of the marrow.

### CASE REPORTS

*Case 1* was a 32 year old white married female who presented herself on July 23, 1946 because of bleeding from the gums, vagina and under the skin for 3 weeks.

At the age of 5 she had had uncomplicated measles and chickenpox. At 12 she began to menstruate, the periods occurring every 21 to 28 days and lasting 2 days. Three years later she had a tonsillectomy which seemed to interrupt a series of moderately severe sore throats. At the age of 20 she had a trichomonas vaginitis and at this time noted that certain areas on her body, especially the arms, were losing their normal pigmentation. This depigmentation was not accompanied by pain, pruritus or local irritation. She married at this time and for the next 4 years tried unsuccessfully to become pregnant. At the age of 25 she began to complain of joint pains. At first the shoulders were involved and after aching for several days the pain passed to the elbows, wrists, knees and small joints of the fingers. These joints subsequently became hot, tender, and swollen. She visited various clinics seeking relief of her joint pains but received little benefit. At about this same time it was found that her uterus was slightly deviated to the left and the right fallopian tube was occluded. By the time she was 27 her joint pains had become more severe, and pain and soreness were present most of the time. She now began to receive shots for her arthritis and after a year seemed to feel much better. At 28 and again at 31 she had exacerbations of her joint symptoms. The shots for the arthritis were discontinued in January 1946. In the fall of 1945 she became aware of the fact that a spot on the top of her head, which had been present for about 8 years and had never bothered her, began to appear inflamed. Her physician prescribed an ointment which had little effect. At the same time she noticed a purplish red rash on both cheeks and the bridge of the nose. However this too had been present, more or less, for 4 years. The rash never caused pain or itching, was always dry, and was darker in color at the time of the menstrual periods. A dermatologist diagnosed the lesion as lupus. She was advised to keep out of the sun and in November 1945 began to receive injections of gold 50 mg per injection every 5 days. These were continued until July 1946, with progressive improvement in the rash.

At the time of her admission to the hospital she stated that she had begun to bleed about 14 days previously. She had no pain but noted that she was weaker than normally and tired more easily. The vaginal bleeding, present for 12 days, and at first consisting only of thin blood, now contained clots. She had been given a shot to stop the vaginal bleeding without effect. One week before admission she developed a fever (which went as high as 102.8°F) and 2 days later her throat became sore. She was given another shot to stop the bleeding and sulfa pills for the sore throat. The sore throat improved but the vaginal bleeding continued, as did the bleeding from the gums. Slight trauma caused the appearance of large black and blue spots on the skin. The only other symptoms of significance were a weight loss of 5 pounds in the preceding 2 weeks and headaches for the preceding 5 days.

On examination she was found to be pale but did not appear seriously ill. There was a nickle sized area of alopecia at the vertex of the skull. The skin was both pigmented and depigmented in various places on both cheeks. Over the bridge of the nose she had a so called butterfly distribution and was more red over the nose than elsewhere. The sclerae were clear, the conjunctivae pale, and funduscopic examination revealed no hemorrhages or abnormal findings. There was a hemorrhagic area on the right buccal mucosa. The gums were soft, friable, and bled upon slight pressure. There were palpable nontender cervical, and

tender submental lymph nodes. The blood pressure was 118/60. The apex of the heart was 8 cm from the mid-sternum in the fifth interspace, and a systolic murmur was heard at the apex and base. There were no palpable organs or masses in the abdomen but there was slight tenderness on deep and superficial palpation in the epigastrium and right lower quadrant. Many bluish ecchymotic areas were seen on all extremities. Many of the small joints of the hands were collared with small hard nodules which seemed to be outgrowths from underlying bone. There were depigmented areas on both arms and legs. The reflexes were normal. Vaginal examination disclosed tenderness in the right cul-de-sac and retroversion of the uterus.

On July 24, 1946 the red cell count was 1.73 million, the hemoglobin 20 per cent (3.1 Gm), and the white cell count was 1,750. There were 2 polys, 95 lymphs, 1 monocyte, 2 eosinophils. Toxicity was 2 plus and poikilocytosis 3 plus. The reticulocyte count was 0.1 per cent and the platelet count was zero. Bleeding time was in excess of 20 minutes while coagulation time was 3 minutes. Total plasma protein was 6.3 grams per 100 cc, carbon dioxide combining power was 29 volumes per cent, chlorides 630 mg of NaCl per 100 cc, and Kahn test was negative. The electrocardiogram showed a sinus tachycardia of 110 with an abnormal, though nondiagnostic, configuration. The urine was alkaline in reaction with a specific gravity of 1.020, 2 plus albumin, 3 plus occult blood, and no sugar, acetone or bile. The marrow obtained from the sternum was, for the most part, replaced by fibrous tissue. Only an occasional normal marrow cell was present. The cellular elements which constituted what remained of the marrow were lymphocytes and plasma cells. The findings were typical of aplastic anemia with fibrosis of the marrow.

The day after admission she was started on 20,000 units of penicillin every 3 hours and the following day she was given 1,000 cc of whole blood. The vaginal bleeding continued and she vomited small amounts of blood. Ten per cent BAL was started in quantities of 2 cc every 4 hours on July 26. In spite of these measures the patient began to bleed profusely from the nose and bilateral nasal packs were required to control the bleeding. Eight cc of 10 per cent BAL was given that day supplemented by 500 cc of blood. The BAL and penicillin were continued, intravenous glucose, saline, and oxygen were given, and alcohol sponge baths and cold enemas were used, but in spite of these measures the temperature rose to 105.6°F 2 days later.

On July 30, 1946 the patient's condition appeared to be terminal. She was dyspneic and extremely weak. Pulse was weak and heart sounds resembled embryocardia. She expired the same day. Permission for postmortem examination was denied.

*Case 2* was a 59 year old white married female who, on her first admission to the hospital on May 2, 1944, complained of nose bleeds for 4 weeks and the appearance of black and blue spots for 2 weeks. Her history dated back to 1928 when, at the age of 43, she first began to notice pain and swelling in the joints of the index finger of the right hand. In 1932 she had a tonsillectomy following which the arthritis spread to all the fingers of the right hand and to the toes of the right foot. In 1936 her teeth were removed, but still no relief was obtained from her joint symptoms. By this time the small joints of the left hand were also involved. She continued to have pain interspersed with occasional spontaneous remissions and exacerbations until April 1944. At that time she again presented herself for treatment to her physician. She was given liver, iron, concentrated yellow bone marrow, a bland diet, local heat and injections of gold. The exact dose of the gold is not known but she was given about 8 injections in all.

Her past history revealed no serious illnesses or operations. She had 5 pregnancies of which only 2 were carried to term. As a child she had chickenpox, measles and mumps. Menopause occurred at 41. Menstrual flow was always copious. She could not eat fish, strawberries, cherries or ice cream as these foods caused hives. Her mother and father died of pneumonia, otherwise the family history was non-contributory. She had taken cathartics and Anacin for many years.

The present illness began about 4 weeks before admission to the hospital with the sudden onset of a severe nose bleed, provoked by a rapid change of position from lying to standing. Bleeding continued for several hours and the blood came in gushes. One week later she had a recurrence of bleeding. In both instances the bleeding was principally from the right nostril. About this time she also began to have some dyspnea on exertion and a pounding sensation in her head and ears whenever she changed position rapidly or became excited. These symptoms progressed and 1 week before admission to the hospital she began to have ankle edema which accumulated during the day and disappeared at night. Coincidentally with the onset of the nose bleeds, clusters of small reddish points, principally on her

chest, made their appearance. These spots appeared suddenly and disappeared in about 3 days. At times these spots became confluent. About 2 weeks later, larger purplish black and blue spots began to appear, especially on the upper arms. These were not the results of trauma. Several days later, pain in the right eye, shoulder, hip and thigh appeared. The pain was described as a feeling of soreness, was intermittent in character and at times became severe enough to restrict motion. In addition to the above complaints she noted a gradually increasing fatigueability.

On admission to the hospital on May 2, 1944 she was found to be obese and pale. A small ulcer was seen on the soft palate and another in the right tonsillar fossa. The pharynx was injected. The heart was not enlarged, rhythm was regular, and a soft systolic murmur was heard at the apex. No organs or masses were felt in the abdomen. Multiple petechiae and ecchymoses were seen everywhere on the skin.

*Laboratory examination* R B C 1.79 million, Hgb 30 per cent, W B C 5,000. Differential 8 polys, 3 eosinophils, 1 basophil, and 88 lymphocytes. The nonprotein nitrogen was 41 mg per cent and the blood sugar was 148 mg per cent. The urine had a specific gravity of 1.014 and was negative for albumin and sugar. The bleeding time was 8 minutes and the coagulation time 4 minutes, 40 seconds. The platelet count by the direct method was 190,000. Stools were negative for occult blood and the Kahn test was negative.

A diagnosis of hypoplasia of the marrow was made. Sternal puncture revealed a moderately cellular marrow. Megakaryocytes were rare. Erythroid development was normoblastic with evidence of great regenerative activity. Granulocyte development was moderately depressed, but adequate numbers of late granulocytes were present to justify the expectation that the granulopenia was a transient phenomenon. Many primitive cells were encountered. The findings were interpreted as compatible with the clinical impression of hypoplasia of the marrow, in a regenerative phase.

She received several transfusions while in the hospital and following her discharge continued to receive about one transfusion every month. In spite of this she persisted in having an anemia, granulopenia and thrombopenia. She began to have rather frequent febrile reactions following transfusions of whole blood and, because of this, red cell transfusions were often given instead.

By the end of 1945, she had received about 18 transfusions, but her condition was essentially unchanged. She was pale and weak and there were pigmented blotches on her zygoma and over the lips. There were bony deformities and partial ankylosis of the distal and interphalangeal joints of the hands. Her R B C was 2.30, Hgb 55 per cent, W B C 5,400.

On March 30, 1946 she was again admitted to the hospital for study, complaining of weakness and black and blue spots. By this time she had received 33 transfusions. Physical examination was essentially negative except for obesity, pallor, petechiae, and ecchymoses. Laboratory examination revealed the following: icterus index 6, cholesterol 230 mg per cent with 60 per cent esters, total protein 6 Gm with an albumin of 3.7 Gm and globulin of 2.3 Gm. The fasting blood sugar was 104 mg per cent and the nonprotein nitrogen was 38 mg per cent. Urinalysis revealed nothing except a faint trace of albumin. The stool cultures were negative and the basal metabolic rate was -10. She received 7 blood transfusions and a blood count taken before the last transfusion revealed an R B C of 4.5, Hgb 81 per cent, W B C 4,500 with a differential of 30 per cent polys and 61 per cent lymphs. Sternal marrow was now quite cellular but with retarded liberation of mature nucleated red cells and paucity of megakaryocytes. Following this admission she received a therapeutic trial with folic acid (15 mg) and thyroid (130 mg daily).

On September 10, 1946 she was again hospitalized for transfusions, by this time having received 52. Her admission hemogram was R B C 1.94, Hgb 38 per cent, W B C 1,500 with a differential of polys 23 per cent, bands 6 per cent, lymphs 54 per cent, monocytes 17 per cent, platelets 30,000.

The patient was last seen on January 17, 1947. Soon afterwards she became ill at home and died suddenly. No postmortem examination was performed.

## DISCUSSION

The treatment of toxic reactions secondary to heavy metal therapy had been largely symptomatic until World War II, in spite of the fact that much investigative work was carried out in an attempt to discover effective antidotes for heavy

metal intoxication Following the introduction of BAL (British Anti-Lewisite, 2,3,—dimercaptopropanol) as an antidote for lewisite gas it became effectively used in the treatment of arsenical reactions occurring in the course of antiluetic therapy These results stimulated the investigation of its effectiveness in the treatment of poisoning with other heavy metals and the drug was subsequently found to be similarly effective in counteracting reactions due to mercury, zinc and copper Several publications have appeared of late reporting excellent results with BAL treatment of gold reactions Before administering BAL to man Ragan and Boots<sup>11</sup> gave gold and BAL injections to rats to determine if the newly formed compound of BAL plus gold was toxic They were prompted to do this by the report of Waters and Stock<sup>12</sup> that the compound of cadmium and BAL was toxic Ragan and Boots were not able to demonstrate a similar toxicity Of their 5 cases of dermatitis due to gold there was rapid recovery in 4 following the exhibition of BAL It is of interest to note that the urinary excretion of gold was increased following the administration of BAL in all 5 cases The damage in the fifth case was apparently irreversible Cohen et al <sup>4</sup> also reported 5 cases of gold toxicity with dermatologic manifestations treated with BAL They felt that the results were good in all cases Lockie et al <sup>9</sup> reported 2 cases of hematologic reactions due to gold, one a thrombocytopenic purpura and one a granulocytopenia, in both of which BAL was successfully used

There have been no previous cases of aplastic anemia due to gold reported in which BAL was used The failure of the drug to help in Case 1 above does not imply a condemnation Aplastic anemia in itself has an extremely high mortality Besides this over 25 days had elapsed since the last gold injection had been given and the patient was hardly alive long enough to test the drug's efficacy BAL should certainly be given an adequate therapeutic trial when another case of aplastic anemia due to gold presents itself

#### SUMMARY

Two cases of aplastic anemia following gold therapy are presented, bringing the total number of reported cases to 20 In one of the cases BAL was unsuccessfully used therapeutically

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# METHOD

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## SPINOUS PROCESS PUNCTURE A SIMPLE CLINICAL APPROACH FOR OBTAINING BONE MARROW

By J PHILIP LOGE, M D

**A**RINKIN<sup>1</sup> in 1929 first described sternal puncture as a practical method for cytologic study of hematopoietic activity and since then this simple procedure has been gaining increasing importance as an aid to the clinician

Other sites for obtaining bone marrow had previously been described Caronia<sup>2</sup> in 1922 found the aspiration of marrow from the tibia in children to be practical Nordenson<sup>3</sup> found it possible to obtain marrow by needle puncture from the head of the tibia and femur, the crest of the ilium, the manubrium or body of the sternum, the ribs and the vertebrae In 2 adults with normal peripheral blood pictures he compared the differential cell counts of marrow from the sternum with that from the vertebrae, ribs, pelvic bone, and tibial epiphyses He found agreement in all but the latter, the tibial epiphyseal marrow proving to be inactive Postmortem examinations of marrow obtained within five hours after death were carried out by Stasney and Higgins<sup>5</sup> in 14 normal persons following accidental death Differential counts of sternal, vertebral and rib marrow cells agreed satisfactorily

Recently interest has been focused on the iliac crest as a site for marrow aspiration by the work of Rubinstein <sup>4\*</sup> He studied the results of simultaneous sternal and iliac aspirations in over 200 normal and abnormal individuals, and believes that the procedure of iliac aspiration is simple, that the marrow obtained represents a cytologically active hematopoietic tissue He comments that in diseased states the iliac marrow has at times been much more informative than the sternal marrow

It is the purpose of this paper to describe another site for obtaining marrow by aspiration in adults, namely the lumbar vertebral spinous processes The technic of spinous process puncture is described, and myelograms of marrow collected simultaneously from the sternum and the spinous process are presented for comparison

It is probable that Katsunuma in 1941 (Japan) first used the spinous process puncture as a practical hematologic procedure and it was later adopted by Nakao at the Tokyo Imperial University † In the latter's hands it was preferred to the sternal approach for routine marrow aspirations

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\* Iliac crest punctures were apparently first described by Van den Berghe of Belgium (Van den Berghe and Blitstein *Presse med*, Aug 4, 1945, p 419) *Editor*

† According to Van den Berghe and Blitstein (*Presse med*, Aug 4, 1945, p 419), A and C Heidenreich (*Prensa Med Argent* 12 2818, 1936) first introduced spinous process puncture, later used more extensively by de Weerdt (*Rev Belge Sc Med* 11 297, 1939) *Editor*

## TECHNIC OF SPINOUS PROCESS PUNCTURE

The spinous process of the lumbar vertebrae to be used for marrow aspiration is chosen. We selected L<sub>3</sub> or L<sub>4</sub>, depending on which presented the broadest spinous process. If the patient sits up the spinous processes are easily outlined with the fingers. In slender individuals the topographic details can be observed without



FIG. 1. KLIMA-SCHLEICHER STERNAL PUNCTURE NEEDLE WITH AN ADJUSTABLE GUARD WHICH ALLOWS PRESSURE TO BE APPLIED NEAR THE NEEDLE POINT

even superficial palpation. An attempt should be made to obtain as much convexity of the lumbar region as possible to make the spinous process more prominent.

The area over the selected site is prepared with a 1-1000 solution of tincture of merthiolate and then infiltrated with a 2 per cent solution of procaine. Then an attempt is made to infiltrate the periosteum over the spinous process.

A Klima-Schleicher sternal puncture needle (fig. 1) with the guard set at about  $1\frac{1}{2}$  cm. is pushed through the skin and fixed with a rotary motion in the spinous process, midway between the upper and lower border. The needle, with firm pressure, is advanced at a  $90^\circ$  angle into the skin until it is firmly fixed to the bone (figs. 2 and 3). The obturator is now removed and a dry tight syringe is used to aspirate the marrow. The patient sometimes complains of an uncomfortable sensation as the marrow is being withdrawn. Occasionally at  $1\frac{1}{2}$  cm. the needle is not firmly fixed, and it is then necessary to reset the guard for a greater depth. This



occurs commonly in patients with heavy musculature or obesity. Rarely, though the needle is firmly fixed, aspiration fails to produce a marrow sample, the needle is then advanced slowly a few millimeters at a time until marrow is obtained. At



FIG. 2. KLIMA-SCHLEICHER NEEDLE FIXED IN THE SPINOUS PROCESS OF THE THIRD LUMBAR VERTEBRA

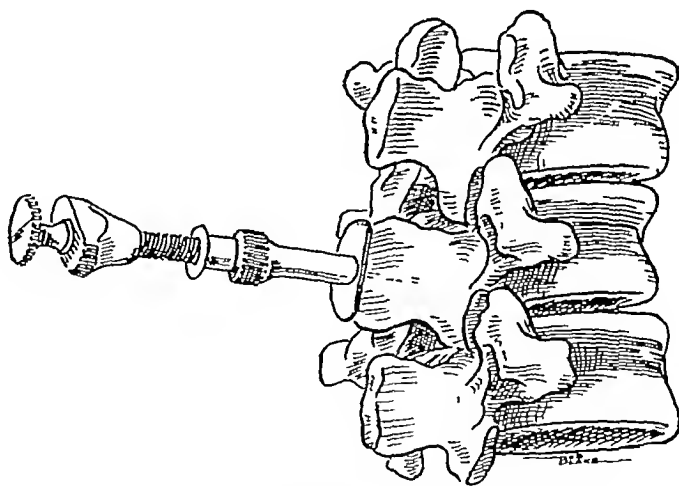


FIG. 3. LUMBAR VERTEBRAE 2, 3, AND 4 WITH THE KLIMA-SCHLEICHER NEEDLE FIXED IN THE SPINOUS PROCESS OF L<sub>3</sub>

no time in our experience was it necessary to go beyond a depth of  $2\frac{1}{2}$  centimeters.

Occasionally, some difficulty is encountered when attempting to fix the tip of the needle in the presenting crest of the spinous process. This difficulty is minimized by (1) selecting the spinous process with the broadest surface, and (2) using the Klima-Schleicher needle which makes it possible to apply pressure and direc-

tion near the needle point. In the hands of the author spinous process puncture was not easily accomplished with the Osgood B-D needle or needles of a similar type where no guard is provided near the needle tip.

The marrow cavity of the spinous process is quite limited when compared to that of the sternum. However, an adequate marrow specimen for cytologic study is readily available. There is some variation in the amount of marrow obtainable, depending on several factors: (1) the position of the needle point in the marrow cavity, (2) the size of the anatomic space, and of course (3) the cellularity of the marrow itself. The quantity of marrow aspirated in the series presented here was 0.1 - 0.4 cc, and was easily available in each case.

### CLINICAL MATERIAL AND METHODS

Twenty-five patients were selected who were hospitalized for nonspecific upper respiratory infections. Their ages ranged from 17 to 75, 80 per cent of the patients were between 17 and 29.

The spinous process puncture was performed first. Immediately thereafter an equal quantity of marrow was obtained from the sternum opposite the second interspace with an Osgood B-D needle of the same gauge. Following each puncture, cover slip or slide smears were made directly and later stained with either a Wright-Giemsa or Wilson stain. Total nucleated cell counts were done using the unoxalated marrow obtained. Marrow reticulocyte counts, using the brilliant cresyl blue method, were completed in 9 of the 25 cases.

After both spinous and sternal marrow samples had been obtained, a blood sample was drawn by puncturing the finger so that a white blood count, differential cell count, Sahli hemoglobin, and reticulocyte count could be checked. A reticulocyte count was made in 9 of the 25 patients studied.

Hemograms of both sternal and spinous marrow samples were performed (table 1) and 500 nucleated cells were counted in each smear, following the criteria for cytologic identification outlined by Wintrobe.<sup>6</sup>

### DISCUSSION

Marrow samples were obtained, in equal quantity, from the spinous processes of L<sub>3</sub> and L<sub>4</sub> and from the sternum opposite the second interspace. The hemograms, based on 500 nucleated cells, were analyzed and compared favorably. It is true that attempts at accurate quantitative determinations of marrow cellularity have been disappointing, and the procedure of routinely obtaining total nucleated cell counts has been abandoned by some hematologists. Total nucleated cell counts, nevertheless, furnish an estimate of marrow cellularity.

Table 1 lists the essential hematologic data obtained in each case. The differential counts of the peripheral blood samples are not included.

It is important to note that in the two oldest patients, aged 72 and 75, the cellularity of the spinous marrow was equal to that of the sternal sample and that the hemograms were almost identical. This suggests that the marrow of the vertebral spinous processes remains an active hematopoietic center in the advanced age group, an observation, to our knowledge, not previously recorded.

TABLE 1 — Ten representative paired hemograms of simultaneous marrow samples from the sternum (st) and lumbar spinous process (sp) of L3 or L4 The peripheral blood data is included

Case number	Age	RBC (millions per cu mm)	Hemoglobin % (Sahl)	WBC (thousands per cu mm.)	Reticulocyte % (peripheral blood)	Marrow sample	Nucleated cells (thousands per cu mm)	Reticulocyte % (marrow)	Myeloblasts	Promyelocytes	Myelocytes (neutrophilic)	Myelocytes (eosinophilic)	Myelocytes (basophilic)	Metamyelocytes (neutrophilic)	Metamyelocytes (eosinophilic)	Metamyelocytes (basophilic)	Neutrophils	Eosinophils	Basophils	Lymphocytes	Reticulum cells	Monocytes	Pronormoblasts	Normoblasts	Plasma cells
15	22	4.65	95	8.58		St	65.2		0.2	1.6	15.8	0	0	31.2	0.4	0	13	1.0	0	11.8	0	0	0.4	23.4	1.2
						St	63.7		0	1.4	11.6	0	0	40	0	0	13.2	0.8	0	15	0	0.2	0.2	17.0	0.6
16	18	4.54		8.95	0.9	Sp	80.2	8.4	1.2	1.0	18.4	0.2	0	41.2	0	0	16.8	0	0	4.8	0.4	0	1.0	15.0	0
						St	115.2	8.1	0.6	2.6	23.2	0.4	0.4	36.0	0.6	0	12.4	0	0	4.2	0.2	0.2	0.4	18.2	0.6
17	75	4.05	80	7.95	0.7	Sp	12.6	1.9	0	0.8	11.4	0	0	14	0.8	0	57	0	0	8.4	0.2	0	0.2	6.8	0.4
						St	13.2	1.8	0.4	1.2	21	0.2	0.2	12.4	0	0	50	0	0	8.2	0.4	0.2	0.2	5.0	0.6
18	55	4.25	85	7.96	0.8	Sp	30.7	3.8	0.2	1.0	8.4	0.6	0	26.0	0.8	0	43	1.0	0	7.0	0	0.4	0.2	9.8	0.6
						St	34.1	4.1	0.8	0.6	20.8	0.2	0	24.0	0.4	0	30.2	1.8	0	8.4	0.4	0.4	0.6	10.0	0.6
19	72	4.58	90	8.95	1.2	Sp	65.1	4.2	0.4	0.4	13.6	0	0.2	16.6	0	0	45	1.0	0.2	10	0	0.4	0.4	11.4	0.4
						St	63.4	8.4	0.6	2.4	14.2	0.6	0	16.4	1.6	0.2	32	1.0	0	10.6	0	0.8	0.4	19.2	0
20	17	4.65		8.15	0.3	Sp	87.1	6.8	1.4	1.2	29	0.4	0	29.0	0	0	11.2	0.4	0	1.8	0.4	0.8	0.2	23.8	0.4
						St			0.6	0.4	30	0.2	0	22.6	0	0	17	0	0	1.4	0.2	1.0	0.8	25.4	0.4
22	19	4.75	100	9.80	0.9	St	12.5	8.3	0.4	1.0	11.0	0	0	45.6	0.2	0	25.2	0	0	9.0	0	0.2	0.6	6.8	
						St	115.3	9.3	0.4	1.4	13.0	0	0	56.6	0.6	0	14.0	0	0	7.6	0	0	0	6.4	
23	52	4.56	90	11.90	0.8	Sp	82.1	3.6	0	1.8	12.0	0	0	26.0	0.4	0	29	0.6	0	16.8	0	0	0	9.0	3.0
						St	67.7	4.2	0.4	1.4	15.0	0	0	20.0	2.2	0	29	2.8	0.2	10.8	0.4	0	0	12.6	3.6
24	19	4.42		7.95	1.1	Sp	120.4	6.5	0.4	2.4	12.0	0.4	0	40	0.6	0	26.8	1.4	0	8	0	1.0	0	7	0
						St	126.2	7.9	0.8	3.2	20.4	0.2	0	35.0	0.2	0	16.2	1.4	0	6	0	0.4	0.6	15	0.6
25	29	4.40	90	7.30	1.2	Sp	125.0	7.3	0.2	0.4	10.2	0	0	36.0	0.4	0	27.6	0	0	9.0	0.2	0.4	0.2	15.4	0
						St	130.0	6.5	0.2	1.0	7.6	0	0	48.4	0.2	0	20.2	0.4	0	11.8	0.2	0	0	10.0	0

The marrow space available for aspiration in any lumbar spinous process is admittedly small. Spinous processes L2, L3, and L4 were obtained from 2 autopsies and subjected to gross examination and some apparent variation was observed in the marrow volume of the vertebral spinous processes of L2, L3, and L4 in the same patient. The variation was greater when the material obtained from the 2 autopsies was compared. Figure 4 shows a median section of L2 from a 75 year old male. Red marrow can be seen in the spinous process and body of the vertebra. No detailed pathologic study was made of the autopsy material as the desired objective was to secure a gross estimate of the size of the available spinous process marrow cavity.

Often, from the technical standpoint, less difficulty was encountered than with the sternal approach. The explanation seems to lie in three factors (1) some pa-

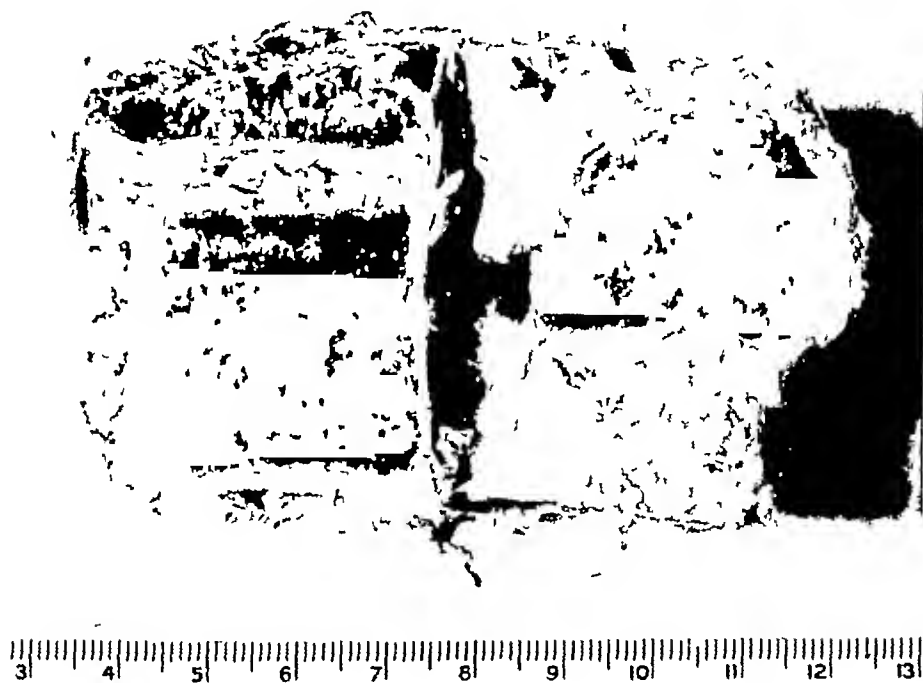


FIG 4 MEDIAN SECTION OF VERTEBRAL BODY AND SPINOUS PROCESS OF L2 FROM A 75 YEAR OLD MALE. RED MARROW IS APPARENT IN THE SPINOUS PROCESS AND BODY OF THE VERTEBRA.

tients complained of less pain during the spinous process puncture, (2) the psychologic value of doing a procedure out of vision of the patient is important, and (3) the avoidance of the disturbing pressure over the less fixed sternum is helpful. A conscientious attempt was made in each case to infiltrate the sternum and spinous process periosteum with procaine. When questioned, 13 patients preferred the spinous approach, 7 the sternal, and 5 gave no choice.

The spinous process puncture is not offered as a method to supplant the sternal route but rather as another technically simple and hematologically reliable way for the clinician to obtain active marrow. The procedure requires no special skill or equipment other than the use of the Klima-Schleicher needle or one of a similar type. When multiple marrow samples are desired the site for puncture can be varied.

It is possible that some patients with a normal cytological sternal marrow pattern may show a pathologic marrow, when such material is obtained from the spinous process

### CONCLUSIONS

- 1 The puncture of a vertebral spinous process for the purpose of aspirating bone marrow, is technically a simple procedure
- 2 Hemograms of marrow samples secured simultaneously from the sternum and spinous process of L<sub>3</sub> or L<sub>4</sub> in 25 patients corresponded well
- 3 The cellularity of the spinous process marrow was found to vary insignificantly from that of the sternal marrow
- 4 Spinous process puncture provides the clinician with another approach for obtaining active marrow by aspiration, rivaling the sternal route in simplicity

### ACKNOWLEDGMENT

The author wishes to express his appreciation to Mr Edmund E. Malecki for his technical assistance

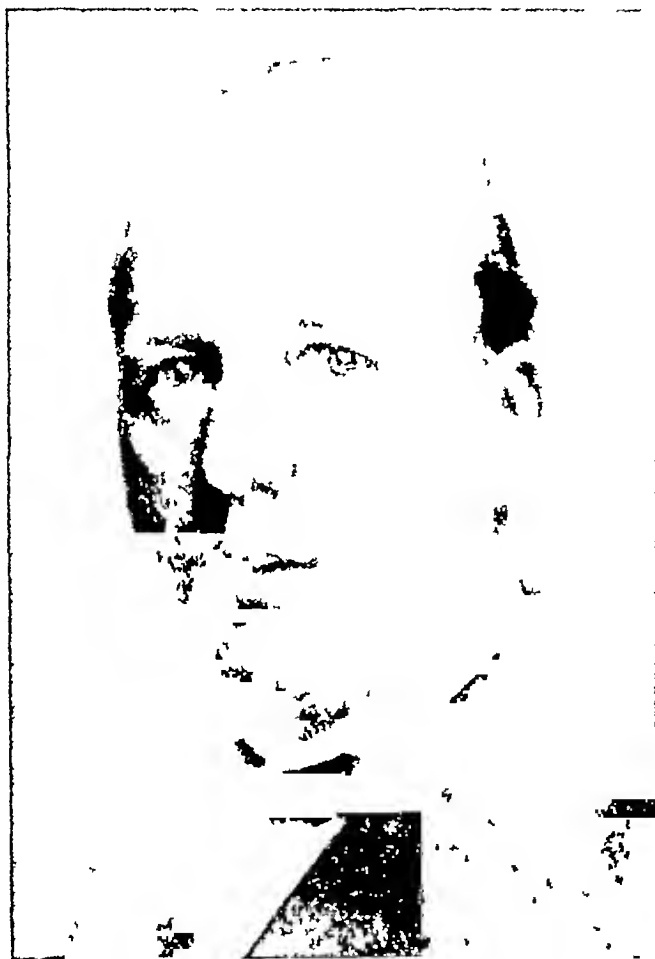
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## ADOLFO FERRATA

(1880-1946)

IT IS now a little over a year since Adolfo Ferrata, the great Italian hematologist, died in Pavia where he was Professor of Clinical Medicine. Ideas, methods of research, diagnosis and therapy have changed and progressed enormously from the days when Ferrata started his activity in the field of blood diseases. However, his contributions to the knowledge of blood morphology and



ADOLFO FERRATA

his work as a writer of several textbooks of hematology deserve to be recalled now that he is no longer with us. His reputation was international.

The first hematological work of Ferrata was done after a brief period of activity with J. Morgenroth, under the direction of, and in collaboration with, A. Pappenheim, in Berlin (1910-11). The investigation of the blood cells at that time was strongly influenced by the work of Paul Ehrlich, who had introduced the first staining methods for blood. Pappenheim and Ferrata, opposing the dominant opin-

ions held by Ehrlich, Pinkus, Lazarus, Naegeli, Denys, Levaditi and with minor variations by Grawitz, Weidenreich and Loewit that the "great mononuclear" was the first morphological step in the evolution of the 'polynuclears,' were able to demonstrate that the 'mononuclear cells' of Ehrlich were an independent strain of white cells, and they introduced for these cells the denomination, thereafter universally accepted, of "monocytes" (1910)

The study of the origin of the monocytes led Ferrata to confront the baffling problem of the genesis of white cells in general. In a previous paper he had agreed with Pappenheim on the genesis of the monocytes from the lymphocytes (1910). Soon after, however, Ferrata modified his views, and showed that a nongranulated immature cell, of lymphocytic appearance, may be found in the bone marrow and in the lymph nodes. He believed that this cell was the common progenitor of the red cells, the white granulated cells of the marrow, the monocytes and the lymphocytes. For this stem cell he created the name "hemocytoblast," from then on used by the majority of the hematologists, although not always with the same significance. His hemocytoblast in the marrow is morphologically indistinguishable from that in the lymph nodes, but it is different in its potentiality of maturation into respectively a medullary white cell, a red cell or a lymphocyte. He was thus recognized as a "neounitarian" among the theorists of hematology. The hemocytoblast of Ferrata (1912-1918) is the "free primitive blood cell" of Cunningham, Sabin, and Doan (1925). Although legitimate doubts may be raised as to whether or not hemocytoblasts, morphologically identical to those of the bone marrow (nongranulated myeloblasts), are to be found in the normal lymph nodes of the adult, it is a fact that a differentiation between the stem cells of the medullary elements and those of the lymphocytes is not easy, as everyday experience in the leukemias with highly undifferentiated elements shows. Nowadays clinical hematologists have found an easy but not very scientific way out of these difficulties: they call the immature nongranulated cells 'blasts'.

The progress made in the knowledge of blood morphology and blood genetics could not fail to influence conceptions of blood pathology. Panchromatic staining methods for blood cells had been proposed since the turn of this century (Jenner 1899, Wright 1902, May-Grunwald 1902, Giemsa 1902) and with the aid of this technic it had been almost universally recognized that the cells present in the peripheral blood of leukemic patients were to a far greater extent immature rather than abnormal cells. On the other hand Banti had maintained that leukemias are neoplastic diseases, not fundamentally different from sarcomas and had defended his thesis with anatomopathological evidence in his *Handbook of Pathological Anatomy* (1907) and in a report to the Italian Pathological Society (Pisa 1913). In the discussion which followed this report, Ferrata and Micheli, two young men at that time, rose to oppose the view of Banti, who recognized, at least in part, the validity of their evidence. In the last years of his life Ferrata was no longer so sure of the essential character of leukemias as hyperplastic processes to be differentiated from true neoplastic diseases, and in recent years some of his pupils even advanced doubts about the systematic nature of the various leukemic lesions, un-

earthing ideas stubbornly defended by Banti in the past *Multa renascentur quae jam cecidere*, indeed! All this is good testimonial of the mental alertness, scientific honesty and intellectual elasticity of Ferrata

The name of the Italian hematologist has remained bound to a special type of cell which many authors, after Naegeli, have called the Ferrata cell. According to Ferrata, these elements, which he first saw in leukemic blood (Ferrata and Franco 1919, Ferrata 1924), represent undifferentiated (multipotent) connective tissue cells. Ferrata called them hemohistioblasts, implying that they were cells capable of an evolution into both connective tissue cells and blood cells. Most hematologists share his opinion that connective tissue cells with a capacity for an evolution in both directions really exist. They are the reticulum cells of the blood-forming organs and possibly of other more widely distributed cells (adventitial cells of the blood capillaries, etc.)

The significance of the particular elements described in the peripheral blood by Ferrata under the name of hemohistioblast has been strongly debated and their existence as such even denied by some authors (Ringoen, Lambin). The writer showed that cells like those described by Ferrata in leukemic blood are also found in the bone marrow of normal human subjects and of mammals in general (1928). That they are not artifacts, i.e. damaged promyelocytes and myelocytes, as postulated by Ringoen and Lambin, is proved by their size (which in many cases is far larger than that of the promyelocytes or myelocytes), their nuclear structure and particularly by the fact that large elements with cytoplasmic pseudopodia, with two blown-up nuclei, with two or three nucleoli, may be seen also in bone marrow sections, often with specific granulations in the cellular body. They represent a form of early differentiation of the reticulum cells into granulocytic elements. This writer, however, has shown that their cellular characteristics are not those of the fixed, pluripotent cells of the connective tissue (reticulum cells), which are the hemohistioblasts in Ferrata's sense. Both in the second edition of his book (1933) and through the work of one of his pupils (Villa 1929), Ferrata accepted this point. Thus it should be recognized that Ferrata not only created an appropriate name expressing a correct idea (hemohistioblast) but that he gave also the first description of a hitherto undescribed type of evolution of the reticulum cell. Similar stages of direct differentiation of the reticulum cells into megaloblasts have also been described by the School of Ferrata in pernicious anemia and acute erythremia (Di Guglielmo).

The contributions of Ferrata are almost totally in the field of morphological hematology. His influence in this direction on a large number of Italian and foreign hematologists, especially those of Spanish and Latin-American origin, was very pronounced. The origin of the blood cells and the relationships between the different cell types were submitted to a minute investigation under experimental conditions, in both normal humans and pathological material by Ferrata and these workers. Cases of blood disease were studied with an almost exclusive interest for the morphology of the blood cells in the peripheral blood, in the bone marrow, etc. Although the achievements in this field were by no means slight, hematology



has a wider scope than pure cytological investigation. And yet Italian hematology has a tradition also of the physiologic approach to the study of blood diseases, from Bizzozzero, Marchiafava, Banti (whose long research on hemolysis is comparatively unknown) down to Zoja, Micheli, Mino, Greppi, Lattes, to name only the better known men. This extreme interest in the morphologic side of blood disorders was very evident in the work of hematologists of many nations at that time. This may have been due to economic limitations which hinder more expensive methods of research. It certainly had a reason in the past. The evidence at hand, however, is to the effect that, although an exact knowledge of blood morphology is a necessary tool for any good hematologist, future progress in the understanding of blood diseases will come from the physiologic approach to these problems.

The activity of Ferrata as a writer of monographs and handbooks started very early with the "Morphology of the Normal and Pathological Blood" (1912). This work was the forerunner of a more extensive handbook "Blood Diseases" (Le Emopatie, 1918-23) which, clearly written and magnificently illustrated with lithographic colored tables (by R. Sarno), had a tremendous success among physicians familiar with the Italian language in Europe and in Latin America. Undoubtedly this book exercised a very great influence in developing interest in hematology in Italy. In the period between the two World Wars, Ferrata in conjunction with several Italian authors (Di Guglielmo, Villa, Introzzi, Greppi, Artom, and others) prepared a new edition in five volumes, which appeared between 1933 and 1935. It is to be regretted that this book is not more widely known. Ferrata contributed a monograph on "The Diseases of the Endocrine Glands" for the Italian "Handbook of Internal Medicine" (Milan 1931), and a "Manual of Blood Diseases," compiled in collaboration with his pupil E. Storti, has just appeared (Milan 1946). This manual is also liberally illustrated with beautiful plates.

Ferrata had a long career as a teacher. In 1922 he was named Professor of Medical Pathology at the University of Messina. He went to Siena in 1923 and the following year he was called to Pavia as Professor of Clinical Medicine. He created a School of Hematology and left behind a distinguished group of scientists, many of them now at the head of several university institutes.

Clear minded and warm hearted, Ferrata was a strong personality and exercised a powerful influence beyond the sphere of his immediate activity. Both in his clinical lectures, as when speaking in medical meetings and congresses, his word was convincing and his intervention clarifying. His powerful physical build, the warmth of his Lombard elocution (he was born in Brescia, a city midway between Milan and Venice), the evident fairness which characterized his approach to any controversy, all contributed to create an atmosphere of sympathetic agreement around him. Although not entirely exempt from the nepotistic habits prevailing in the Italian academic world Ferrata showed himself ready to help young men coming from other schools in the realization of their academic aspirations.

M. VOLTERRA, New York

## EDITORIAL

### MARROW BIOPSY TECHNIQS

DESCRIPTION by Loge in this issue of the spinous process aspiration biopsy of the marrow brings to mind the great development in the use of the marrow puncture as a diagnostic aid in hematologic problems. Although marrow biopsies had previously been occasionally performed, it remained for Seyfarth in 1923 to introduce the sternal approach. The marrow cavity was entered by means of a small trephine and sections of the button of bone, together with curettings, made directly from the marrow cavity were obtained. Arinkin's method of puncturing the sternum by means of a stilette needle and aspirating a small amount of marrow material proved far more flexible and readily adapted to the clinic. Numerous needles and methods of handling the aspirated material have been recommended, but it is likely that the simplest needle and the least handling give the best results, providing that a small amount of material (no more than 0.5 cc) is aspirated. The sternal puncture has in many respects revolutionized diagnostic hematology, so much so that in many clinics, a case is considered inadequately studied if the marrow puncture has not been performed.

The marrow puncture finds its greatest diagnostic use in conditions of pancytopenia, in splenomegalic disturbances, and in thrombocytopenia. Pancytopenia is more often due to aleukemic leukosis, multiple myeloma, and lymphosarcomatosis than to hypoplasia and aplasia of the marrow. The diagnosis of the leukocytic neoplastic diseases as they invade the marrow is usually readily made by marrow puncture, even when the blood picture is completely noncontributory. This is particularly true of multiple myeloma. In many conditions with splenomegaly, the marrow puncture may be distinctly helpful, as for example, in Gaucher's disease, lymphosarcoma, and the hyperplastic marrow as seen in hypersplenism, etc.

In thrombocytopenic purpura, marrow aspiration is almost obligatory, since in no other way can one be sure that the megakaryocytes are present in normal, increased, or decreased numbers. To remove the spleen when the marrow megakaryocytes are greatly lacking or altogether absent is to invite disaster. Splenectomy should be performed only when it seems reasonably certain that the disorder is idiopathic or hypersplenic and when the megakaryocytes are present at least in normal numbers.

Dry marrow taps are occasionally encountered. This occurs not only with fibrotic marrows but occasionally in the extremely cellular marrows of certain cases of aleukemic leukosis. Under such conditions, it may become necessary to perform a trephine biopsy. In our clinic such necessity occurs very infrequently, perhaps once in fifty punctures. The trephine biopsy gives topographic relationships of marrow to bone, extent of fibrous tissue, etc., but from the standpoint of studying cellular histology, the direct sternal aspiration is unsurpassed. Furthermore, good sections can be made from aspirated material by appropriate fixation.

The need for performing the relatively difficult trephine biopsy has dwindled even more as new avenues for approaching the marrow have been introduced.

Puncture of the iliac crest was introduced a few years ago by Van den Berghe of Belgium and its use emphasized in this country by Rubinstein. Spinous process punctures were apparently first described by A. C. and C. L. Heidenreich in an Argentinian article in 1936, and the Belgian De Weerdts testified as to the good results obtained by this method. The Japanese had evidently been using the spinous process puncture for a number of years before our medical officers in the Pacific learned of it. As stated by Loge, this method has a number of advantages. The patient lies on his abdomen without any exact perception of what is going on, the needle does not go over his "heart," and as a result, there is far less psychic trauma. In addition, the spinous processes are so numerous that serial studies in such conditions as idiopathic thrombocytopenic purpura can readily be made. The method may also be used in infants and very young children, in whom sternal puncture is often quite difficult. The advantage of having at least three sites to puncture has already proved itself in a number of cases in our laboratory. For example, it seemed reasonably certain that a recent patient with splenomegaly, pancytopenia, and a high sedimentation rate had lymphosarcomatosis. Sternal and iliac crest punctures were both extremely hypocellular and unhelpful but the spinous process puncture showed almost a pure culture of lymphoblasts, thus not only making the diagnosis, but foregoing the need for performing a trephine biopsy. Preliminary observations in our laboratory of some 30 comparative sternal, vertebral, and iliac crest punctures indicate that the results are very similar from one site to another and that if there is failure by one method success may be obtained with another.

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# ABSTRACTS

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## ERYTHROCYTES AND ERYTHROCYTIC DISEASE

OBSERVATIONS ON THE NORMAL ADULT HUMAN ERYTHROCYTE *P. Ralph* From Department of Anatomy, Ohio State University, Columbus, Ohio *Anat. Rec.* 98: 489-506, 1947

The human red blood corpuscle was studied with bright- and darkfield microscopy in the untreated and treated condition. There is considerable evidence to indicate that there is a plasma membrane, which remains intact even after hemolysis occurs. This membrane contains certain acetone and alcohol extractable lipids which, according to the author, are demonstrable with Sudan black after saturation of the unsaturated bonds. Ralph has confirmed a previous observation (Cowdry, *N. H. Biol. Bull.* 33: 196, 1917) that mitochondria may be present in immature erythrocytes which he has unfortunately referred to as primitive erythrocytes. After cells had been exposed to neutral red for 15-30 minutes, some of them developed refractive globules which were designated as the 'A' granule. The ability of the red blood corpuscle to segregate basic dyes in newly formed vacuoles was first studied by Israel and Papenheim (*Virchows Arch.* 143: 419, 1896) and has been investigated recently by Dustin (*Bull. Acad. Belg. Cl. Sci.* 28: 230, 1942). These, along with reticulation, mitochondria and the vacuole, are signs of immaturity.

O P J

PROLYTIC ION EXCHANGES PRODUCED IN HUMAN RED CELLS BY METHANOL, ETHANOL, GUIACOL, AND RESORCINOL *E. Ponder* From The Nassau Hospital, Mineola, Long Island, N. Y. *J. Gen. Physiol.* 30: 479-491, 1947

Before red blood corpuscles are hemolyzed there is a loss of potassium with a replacement by sodium derived from the media in which the cells are suspended. When red blood corpuscles were suspended in certain alcoholic solutions it was possible to study large losses of potassium, since the amount of lysis was unusually small. As much as 50 per cent of the potassium may be lost from a cell without greatly affecting the disk-sphere transformation and resistance to osmotic hemolysis.

O P J

OSMOTIC PROPERTIES OF THE ERYTHROCYTE. XII. IONIC AND OSMOTIC EQUILIBRIA WITH A COMPLEX EXTERNAL SOLUTION *M. H. Jacobs and D. R. Stewart* From Department of Physiology, University of Pennsylvania, Philadelphia, and Marine Biological Laboratory, Woods Hole, Mass. *J. Cell & Comp. Physiol.* 30: 79-103, 1947

The problem analyzed and discussed in this paper concerns what happens to the internal composition, the pH, the ionization of hemoglobin, and water content of erythrocytes, if they are placed in a medium containing known quantities of penetrating and nonpenetrating anions, cations, neutral molecules and ionized and nonionized proteins.

O P J

MODIFICATION DE LA RÉSISTANCE OSMOTIQUE DES HÉMATIES PAR QUELQUES CORPS ÉLEVANT LA RÉSISTANCE DES CAPILLAIRES *J. L. Parrot and M. Gabe* *Compt. rend. Soc. de biol.* 141: 363, 1947

The authors study the action of several substances: aesculoside, rutoside, d-epicatechine (which are supposed to possess vitamin P activity) on guinea pigs red cells twice washed with saline. They use a

colorimetric method of measuring the hemolysis and express the results in percentage of the total hemolysis. Rutoside has a very poor solubility in water and it is impossible to obtain a concentration higher than 1.3 per cent. Aesculoside and d-epicatechine are very soluble and higher concentrations were studied. These three substances appear to increase the globular resistance to hypotonic solutions. This action is not stronger with large concentrations than with the very dilute solutions first tested (less than 1.3 per cent for the rutoside). Among the three, the d-epicatechine seems to be the more active as small doses as 2.5 per cent definitely increase the globular resistance. Although the authors do not discuss the specificity of this *in vitro* antihemolytic activity (physical interferences, or pH), these experiments seem worthwhile reporting, any new method of testing the activity of the so-called vitamin P (here, by modifications of the cellular permeability of the red cell) is welcome, since the usual tests for capillary permeability (or resistance) are far from being satisfactory.

J P S

**NORMAL AND ABNORMAL BLOOD-COUNTS ON THE WITWATERSRAND** *G W H Schepers* From University of the Witwatersrand, Johannesburg, South Africa *J Path & Bact* 59 199-208, 1947

The three major factors which may contribute to modifying hematologic values on the Witwatersrand are the 6000 foot elevation, the unclouded skies and the general occupation of deep level mining. The results were obtained by examining blood from 100 males and 50 females who were robust and healthy adults, usually between the ages of 20 and 40 years. Medical students were considered unsatisfactory for such studies. There was an erythrocytosis, increased amount of hemoglobin and a slightly elevated color index. Erythrocytes were slightly smaller in diameter and surface area than those from Europeans living at sea level. Leukocyte level was unaltered. Three hundred cases of macrocytic anemia were analyzed statistically. In view of the high normal counts, a large number of patients would be unrelieved of their symptoms if they were not treated before they had fewer than 4.0 million erythrocytes.

O P J

**MACROCYTIC HYPERCHROMIC ANAEMIA ON THE WITWATERSRAND** *G W H Schepers* Johannesburg, South Africa *South African Med J* 21 568-575 and 605-611, 1947

This study deals with the non-mining population, mostly members of Reef Benefit Society Panels, residing on the Witwatersrand in the vicinity of Johannesburg at an elevation of approximately 6,000 feet. From a potential reservoir of 50,000 individuals, 300 persons (0.6 per cent) were found to have abnormalities of the erythrocyte in the direction of macrocytosis and hyperchromatosis. In this area, 6.0 million cells per cu. mm. and 20.0 grams of hemoglobin per 100 cc. of blood are considered average normal values. The mean values for the cases included in the author's series were as follows: erythrocyte count 4.04 million, hemoglobin 18.0 grams, color index 1.37, erythrocyte diameter 7.8 microns, and erythrocyte surface area 102 square microns. The range of the red cell counts in this group was from 1.0 to 7.0 millions. Considerable emphasis is placed upon the hemoglobin surface concentration index which is a measure of the breathing surface of the blood and is calculated by the formula

$$H S C I = \frac{\text{color index}}{\text{mean erythrocyte surface area}} \times 100$$

In the group of cases under consideration, the mean H S C I was 1.06 compared with the normal mean of 1.57, and this is taken as a measure of the lack of physiologic efficiency of the macrocytes. Anisocytosis was regularly present and often marked, while poikilocytosis was less commonly noted (in contrast to pernicious anemia).

Although response to parenteral liver therapy was very good, no conclusions could be drawn as to pathogenesis or epidemiologic factors. The possible roles of constitutional defects, endocrinopathies and urinary infections are discussed. It is suggested that if certain constitutional types fail to achieve adequate adaptation to high altitudes, hypofunction of hemopoietic tissues may be one result.

L E Y

**FAMILIAL HYPOPLASTIC ANEMIA OF CHILDHOOD** REPORT OF EIGHT CASES IN TWO FAMILIES WITH BENEFICIAL EFFECT OF SPLENECTOMY IN ONE CASE *S Estren and W Dameshek* From the Blood Labora

tory of the J H Pratt Diagnostic Hospital and Boston Dispensary, the Boston Floating Hospital, and Tufts College Medical School, Boston, Mass. *Am J Dis Child* 73 671-687, 1947

This report is concerned with the first recorded instances in which hypoplasia of the marrow has been noted as the sole familial trait. It is pointed out that the closest similar cases in the literature are those of Fanconi, in which hypoplasia of the marrow was one of a number of congenital and familial abnormalities.

Three of 7 siblings in one family and 5 of 14 in another family had apparently identical disorders characterized by peripheral pancytopenia, pallor, weakness, and bleeding tendency. In the 2 cases in which the marrow was examined, there was quantitative hypoplasia of all elements, but the proportions of erythroid cells were somewhat higher than normal. In one of the cases splenectomy resulted in cessation of the hemorrhagic tendency together with some increase in red cells and leukocytes and a more marked increase in platelets. The spleen was not enlarged and showed no abnormalities.

It is the opinion of the authors that in cases of complete or almost complete aplasia of the marrow, and in cases in which a reduction of platelets is accompanied by a reduction of megakaryocytes in the marrow, splenectomy will probably be of little value. When megakaryocytes are present to some degree in the marrow, however, and particularly when lack of platelet production in the megakaryocytes can be demonstrated, and when, in addition, erythroid elements show some evidence of regenerative activity, splenectomy is deemed worthy of consideration. It is suggested that extirpation of a normal spleen under such circumstances removes the regulatory (inhibitory, humoral) effect on red cells, granulocytes and platelets in the marrow.

The concise review presented of hypoplastic anemias of infancy and childhood is of particular interest because of the confusion created by some of the earlier reports on this subject.

L E Y

A CASE OF IDIOPATHIC METHAEMOGLOBINAEMIA TREATED BY ASCORBIC ACID AND METHYLENE BLUE. *E J King, J C White, and M Gilchrist*. From British Postgraduate Medical School, London. *J Path & Bact* 59 181-188, 1947.

Idiopathic methemoglobinemia was studied in a girl aged 25 years who had had a dusky cyanotic hue of the face, hands, lips, ears and fingernails for as long as she could remember. Total hemoglobin was 17.0 Gm per 100 ml. Oxygen carrying hemoglobin was 10.8 Gm per 100 ml. The 36 per cent difference consisted of 6.2 Gm of methemoglobin. Plasma ascorbic acid concentration was low. The optimum amount of ascorbic acid was between 200 and 300 mg per day. Under such therapy the oxygen capacity of the blood rose and cyanosis lessened in three days and the latter disappeared within a week. Similar results were obtained with methylene blue. When treatment was discontinued there was a gradual return to the previous status. In vitro studies indicate a probable defect in the erythrocyte enzyme system.

O P J

BLOOD CHANGES IN THE AGED. *O Olbrich*. From the Biochemical Laboratory, Royal Infirmary, Edinburgh, and Queensberry House Hospital, Edinburgh. *Edinburgh M J* 54 306-321, 1947.

Properly controlled blood studies were made on men and women from 60 to 100 years old. Red cell, hematocrit, mean corpuscular volume, color index and white counts showed completely normal values, with the usual sex difference. These data, in agreement with other reports, indicate the pathologic importance of any degree of anemia in old age.

C A F

## BLOOD GROUPS, THE Rh FACTOR AND BLOOD TRANSFUSIONS

KERNICTERUS. A FOLLOW-UP STUDY OF THIRTY-FIVE ERYTHROBLASTOTIC INFANTS. *R Stiller*. From the Children's Hospital, Washington, D C. *Am J Dis Child* 73 651-662, 1947.

The author points out that with the advent of more detailed knowledge of the pathogenesis and treatment of erythroblastosis fetalis, interest is being focused on possible untoward effects of therapy that may result in a live but helpless infant with kernicterus. Wiener's hypothesis regarding the pathogenesis of kernicterus is cited. According to this concept, agglutinated red cells plug the capillaries of the brain,

causing toxemia. Ganglion cells, being most easily injured by lack of oxygen, die and are then stained by bilirubin.

Follow-up records of 35 cases of erythroblastosis were reviewed. All patients included in the series had had neonatal jaundice or anemia and were Rh positive children born of Rh negative mothers. In the 6 fatal cases no correlation could be shown between severity and extent of clinical manifestations of neurologic involvement and the findings on pathologic examination, only 2 of the 6 showed kernicterus. Of the 29 living children, 4 showed neurologic signs attributed to kernicterus, but in only 2 of these patients was involvement of the central nervous system noted during the neonatal period. These figures are compared with those reported by other observers.

L E Y

RECHERCHE SUR LA CAUSE DE L'ICTERE GRAVE FAMILIAL DES MULETONS. SES RAPPORTS AVEC LA MALADIE HÉMOLYTIQUE DU NOUVEAU-NE. *J. Caroli et M. Bessis*. Centre National de la Transfusion Sanguine et de Recherches Hématologiques, Paris. *Rev. Hemat.* 2: 207-228, 1947.

There is in the Poitou district of France, a disease, well studied by Sausseau, which affects 8 per cent of new-born mules. It is an acute hemolytic jaundice with hemoglobinuria appearing within a few hours after birth and usually terminating in death. Following the time a mare gave birth to a jaundiced mule, all its offspring will die of icterus gravis. But if this mare is covered by a stallion, the colt will always be healthy. These features, so similar to the hemolytic disease of the human new-born, caught the attention of Caroli and Bessis, and these authors sought for a heteroimmunisation of the mare covered by an ass.

The hematologic examination of the new-born mule with hemolytic jaundice, shows a striking autoagglutination of the red cells, spindle-shaped red cells, microspherocytosis. The anatomic findings are the same as those found in rats treated by hemolytic serum. Dilatation of the hepatic sinus, generalized pigmentar infiltration, but no hydrops foetalis and no kernicterus, as often seen in the human new-born disease. The serologic study shows a very high titer of antibodies against the ass and mule red cells in the serums of the diseased mare, while the other mares have only a few or no antibodies. The antibodies often are incomplete (blocking antibodies). Intravenous injections of an ass blood to the mare increases the titer of the antibodies much more in the immunized mares than in the others.

The authors compare this mule disease to experimental hemolytic jaundice, and to the human hemolytic disease. The mule disease is very close to the experimental disease of the rat and differs from the human disease only by the lack of cytotoxic lesions (cirrhosis, nervous lesions). The parasitic etiology, first considered, is eliminated by these immunologic findings concerning the prophylaxis and therapeutics of the Poitou mule disease, which findings may prove of considerable importance to cattle breeders. The study of this animal disease can also lead to a better understanding of the human hemolytic disease of the new-born.

J P S

THE RH FACTOR. *W. S. Stanbury*. From the Canadian Red Cross Blood Transfusion Service, Toronto, Ontario. *Canad. M. A. J.* 57: 363-370, 1947.

The author presents a well-organized summary of the present status of the Rh-Hr system with particular reference to inheritance and isoimmunization. Readers who are relatively unacquainted with the voluminous and confusing literature in this field will find this review helpful. Some authorities would disagree with the rather firm recommendation that early induction of labor be considered when the mother's serum contains a high titer of blocking antibodies.

L E Y

TRANSFUSIÓN SANGUÍNEA Y TIEMPO DE COAGULACIÓN (BLOOD TRANSFUSION AND COAGULATION TIME). *Alberto S. Benchimol*. La Prensa Médica Argentina 34: 3, 154, 1947.

The author reviews the literature favoring direct blood transfusion against the citrated method. Reactions and shock are rare with the former method. He used the metallic apparatus devised by Luis de Marval and the cannula of E. F. Cichetto.

In 20 cases studied, using both the direct and the citrate methods, there were no reactions with the former, and four post-transfusion reactions with the latter. Blood clotting time was definitely

diminished in all cases where the direct method was used, and in only 4 of 10 cases where the citrate method was used

The direct method is, according to the author, the method of choice, whenever blood coagulation time should be shortened preoperatively. It is indicated especially in neurosurgery, in hemophilia, anemias, and hepatic insufficiency

The transfusion should be made twenty-four hours before operation

R M S

#### LOSS OF VIRULENCE OF *TREPONEMA PALLIDUM* DURING PROCESSING OF DRIED BLOOD SERUM T F Proby

From the Biologics Control Laboratory, National Institute of Health, Bethesda, Maryland. Pub Health Rep 62 1199-1202, 1947

A saline suspension of treponemes of the Nichols strain was prepared from rabbit testicles and mixed with normal horse serum. A portion of the mixture was then used for immediate inoculation into control rabbits and the remainder frozen under vacuum in a bath of carbon dioxide and cellusolve for thirty minutes. Drying of the frozen material was then continued under vacuum for twenty hours with the ampules exposed to room temperature, a procedure which had previously been shown to reduce moisture content to less than 1 per cent. One group of rabbits was inoculated with restored dried material soon after completion of the drying cycle, and a second group was inoculated two days later. Dark-field examination of the resuspended material showed some atypical forms of what appeared to be nonmotile treponemes.

All 3 of the control rabbits developed syphilitic lesions within forty-two days, while none of the 6 rabbits inoculated with processed material developed evidence of syphilis during the observation period of one hundred and forty days. Three successive subtransfers observed for thirteen, seven, and 9 months respectively also remained negative.

These observations confirm the results of Turner, Bauer, and Kluth, and offer somewhat more conclusive evidence that the danger of transmitting syphilis is eliminated by proper freezing and drying of plasma or serum. The author points out that Turner has demonstrated that motility and virulence of *T. pallidum* are not altered appreciably by freezing at  $-78^{\circ}\text{C}$  and maintenance of this temperature for at least one year. Turner has also showed that freezing at  $-10^{\circ}$  or  $-20^{\circ}\text{C}$  does not adversely affect treponemes, but that maintenance at these temperatures for two months causes their death. The practical importance of these studies is obvious.

L E Y

## HEMOGLOBINURIA

THE INFLUENCE OF INJECTIONS OF HOMOLOGOUS HEMOGLOBIN ON THE KIDNEYS OF NORMAL AND DEHYDRATED ANIMALS J J Latsch From the Department of Pathology, University of Wisconsin Medical School, Madison, Wisconsin. J Exper Med 86 153-158, 1947

Hemoglobin solutions containing stroma were injected intraperitoneally in single doses into 37 rats (5 to 7 Gm /Kg) and 26 guinea pigs (1 to 3.5 Gm /Kg) which had been fed and offered water up to the time of injection. Microscopic examination of the kidneys following this procedure revealed an occasional cast in only 2 rats. Sections from the remaining 35 rats and from the 26 guinea pigs showed no casts or tubular changes.

Water was withheld from 16 rabbits for periods of one to five days after which stroma-free hemoglobin solutions were injected intravenously at one time or in divided doses on successive days toward the end of the dehydration period. Water was then withheld for a period of eighteen to 24 hours after the last injection. Thereafter varying amounts of water were given daily for one week. The quantity of hemoglobin injected varied from a single dose of 1.3 Gm /Kg to 1.8 Gm /Kg in eight divided doses on two consecutive days. In the 3 rabbits dying prior to the fourth day following hemoglobin injection, the kidneys were congested and a reddish yellow substance of homogeneous glasslike consistency was observed in the tubular lumina. These collections were not considered to represent formed casts. In 12 of 13 rabbits examined from four to forty days after hemoglobin injection, characteristic pigmented casts and associated tubular dilatation were demonstrated. Minimal necrosis of tubular epithelium in 5 of 13 rabbits was thought to have followed, rather than preceded, the plugging of tubules by casts. Lesions in other organs were of no particular significance.



It is emphasized that consistent reproduction of hemoglobinuric nephrosis has not been previously accomplished without preliminary direct trauma to the kidneys, and that there is need for studying the role of dehydration in other species, particularly man. It should be pointed out that urinary pH was not controlled in this study, and that the hydrated control animals were rats and guinea pigs rather than rabbits.

L E Y

## FOLIC ACID

FOLIC ACID Y SubbaRow, R B Angier, et al Ann N Y Acad Sc 48 255-350, 1946

This monograph, comprising a series of thirteen articles by various authors on pteroylglutamic (folic) acid, covers in detail its chemistry, synthesis, pharmacology, physiology, and clinical applications. Published in 1946, it brings together theoretic and clinical investigations available up to the middle of that year. Much of the material has since been recorded in reviews and individual articles elsewhere, especially the description of the synthesis of pteroylglutamic acid and the observations on the patients in the clinic.

It is of interest that the mode of action of folic acid and the absence of marked activity of conjugated forms, are yet controversial. Heinle and Welch (pp 343-346) note the failure of other components of the vitamin B complex, and of biotin, paraaminobenzoic acid, choline, and inositol, to augment the effect of pteroylglutamic acid. The yeast conjugate, they found, was not utilized by human beings, although more recent findings suggest that, in sufficiently large doses, yeast-conjugate is effective (Spies, *Experiments with Folic Acid*, New York, Interscience Publishers 1947, p 101). Nor is conjugated folic acid the extrinsic factor of hematopoiesis. The exact role of folic acid is today, as a year ago, still obscure.

This is a valuable and complete collection of observations on pteroylglutamic acid as of May 1946.  
S E

EL EFECTO DE LOS CONJUGADOS DE ACIDO FOLICO EN EL ESPRÚ (THE EFFECT OF FOLIC ACID CONJUGATES IN SPRUE) Ramón M Suárez Bol Asociación Médica de Puerto Rico 39 8 281 1947

In a lecture given at Clínica Pila in Ponce, Puerto Rico, the author gave the results obtained in 4 cases of sprue treated with conjugated forms of folic acid. Fermentation folic acid in daily doses, equivalent to 3.1 mg of folic acid administered intramuscularly, was used in 2 cases. Bc conjugate in daily oral doses of 7 cc, equivalent to 8.4 mg of folic acid, in one case, and another case of sprue was treated with 2 G in daily oral doses of 20 mg.

Both cases treated with fermentation folic acid showed, not only clinical and hematologic improvement, but also an increased urinary output of free folic acid.

The case treated with Bc conjugate showed a striking clinical and hematologic response, but only a slight urinary elimination of free folic acid, and the case treated with 2 G failed to show definite improvement.

As already reported by Suarez, Welch et al, the urinary output of free folic acid increased when concentrated liver extract was added.

It is stated that the mechanism of action of free folic acid, and of its conjugated forms, is unknown, but that it is definitely established that folic acid is not the intrinsic factor of Castle. It may be that folic acid is somehow related to the extrinsic factor, but until more is known about the biochemistry of the antianemic compounds, premature speculations should be avoided.

R M S

OBSERVATIONS ON THE SPECIFICITY OF THE FOLIC ACID MOLECULE T D Spies, R E Stone, and R O Brundenburg From the Univ of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Ala South M J 40 618-19, 1947

The administration of methyl-folic acid to one patient with sprue and one with pernicious anemia was followed by no changes in the peripheral blood, the bone marrow, or the clinical course of the patient. Administration of ordinary folic acid, on the other hand, was followed by a prompt improvement.

The authors argue from this that the particular chemical configuration known as folic acid is highly specific in its effect on erythropoiesis. It would have been of interest to have included sufficient details

concerning the patients to note whether they became worse, since methylfolic acid has actions antagonistic to folic acid, i.e., it is an 'anti-folic acid material' (Arch Biochem 12 318, 1947). Alterations in the glutamic acid portion of the folic acid molecule do not qualitatively affect its activity, since the triglutamyl and heptaglutamyl compounds corresponding to pteroylglutamic acid are both antianemic. Methylfolic acid consists of an alteration in the pteridine portion of the molecule, and antagonizes the action of folic acid. The significance of this chemical alteration, and the uses of antifolic acid compounds, are yet to be determined.

S E

SOME RECENT EXPERIENCES WITH VITAMINS AND VITAMIN DEFICIENCIES *T. D. Spies and R. E. Stone* From the University of Cincinnati Studies in Nutrition, at the Hillman Hospital, Birmingham, Alabama South M J 40 46-55, 1947

During the course of studies on the antianemic activities of pteroylglutamic (folic) acid, a number of related compounds were tested for their possible similar action. Pteroylglutamic acid itself, and pteroyltriglutamic acid (the fermentation factor, also written pteroyl-diglutamyl glutamic acid) were found to have similar actions in pernicious and related anemias. Pteroyl-heptaglutamic acid (also called pteroyl-hexaglutamyl-glutamic acid, the  $B_6$  conjugate material) was also found to give a good response, contradicting in this regard previous findings by Bethell (Univ Mich Hosp Bull 12 42, 1940) and Heinle (Proc Cent Soc Clin Res 19 27, 1946), who had found the heptaglutamic compound to have no effect. According to Spies, this difference is probably a matter of dosage: the body is able to release folic acid from the heptaglutamic compound only in small amounts, but if sufficiently large doses of the compound are given, a good antianemic effect is obtained.

In contrast, there was no antianemic effect from pterotic acid, glutamic acid, or xanthopterin, all chemically related to folic acid.

S E

STUDIES ON THE PATHOLOGIC EFFECTS PRODUCED BY TWO ANALOGUES OF PYRIDOXINE *C. W. Mushett, R. B. Stebbins, and M. N. Barton* From the Merck Institute for Therapeutic Research, Rahway, N. J. Tr N Y Acad Sc 9 291-6, 1947

Materials which are chemically related but physiologically antagonistic to certain substances have recently become important as displacing agents or anti-substances (paraaminobenzoic acid for sulfanilamide, methylfolic acid for folic acid, antivitamin B<sub>6</sub>). The present report deals with the changes produced in lymphoid and hematopoietic tissues by two antipyridoxine substances, desoxypyridoxine and methoxypyridoxine.

Interesting findings, produced alike by pyridoxine deficiency and by administration of antipyridoxine compounds, included a reduction in the bulk of the spleen due to hypoplasia of the lymphoid elements. In some species of experimental animals, atrophy of the thymus and the lymph nodes was also present. Concomitant with these abnormalities occurred the development of a microcytic hypochromic anemia, leukopenia, and lymphocytopenia and reduction in the erythroid and myeloid elements of the bone marrow.

An important finding in the adrenal glands consisted of a depletion in the lipid content, with an enlargement of the adrenals. The authors, therefore, speculate—since a relationship between adrenal cortex and lymphopoiesis has been suggested—whether the lymphoid atrophy might be the result of adrenal stimulation rather than the result of a direct action of the antipyridoxines. This question has not yet been answered.

Not all findings were present in all species of animals studied. Variations in species response and doubt as to manner of action make it difficult to attempt to translate these results to human experience.

S E

## LEUCOCYTIC DISEASE

THE USE OF BAL (2,3-DIMERCAPTOPROPANOL) IN THE TREATMENT OF AGRANULOCYTOSIS FOLLOWING INTENSIVE ARSENOTHERAPY FOR SYPHILIS *H. L. Hooley* Dept of Medicine, University of Alabama Medical School, Birmingham, Alabama Ann Int Med 27 231-238, 1947

Twelve cases of leukopenia following arsenical therapy are reported. In 7 there was complete absence of polymorphonuclear leucocytes in the peripheral blood and the remainder varied between 5 and 15 per cent. All patients were febrile and 10 showed agranulocytic angina. Treatment consisted of injections of BAL in peanut oil 2.5 mg./kilo every four hours for the first two days, then 1-2 times a day for six days. Penicillin and sulfonamide drugs were not employed. Recovery occurred in all cases and provides further convincing evidence of the effectiveness of BAL in counteracting toxic manifestations of arsenic and other heavy metals.

C A F

UN NOUVEAU CAS D'ADÉNO-LYMPHOÏDITE AIGUË BÉNIGNE À DÉBUT INGUINAL AVEC ULCÉRATION GÉNÉTALE (A NEW CASE OF BENIGN ACUTE LYMPHADENITIS BEGINNING IN THE INGUINAL REGION WITH GENITAL ULCERATION) R. Demanche and P. Lersb. Sang 18 93, 1947

This is the third case published in France of infectious mononucleosis with a genital ulceration and inguinal adenopathy (the others published by P. Chevallier and by Chassagne & Forgeois). The patient, 23 years old, noticed on the first of May a slight genital erosion on the penis, the tenth of May an inguinal adenopathy appeared. The patient was seen for the first time on the sixteenth of May. He was tired, with 38.8°C fever. The genital ulceration had disappeared, but several lymph nodes formed in the left groin. The twenty-eighth of May, the adenopathies were generalized, especially in the cervical area. The volume of the spleen was increased. On the second of June the fever receded. The hematologic examination (twenty-fourth of May) gave the following results: leucocytes, 7400, polynuclears, 35, lymphocytes, 43, monocytes, 23. On the thirtieth of May a Paul and Bunnell reaction was strongly positive serum alone, 2.22.22.210, serum + guinea pig kidney, 2.22.22.210, serum + beef red cells 0.00.00.000. On the twenty-second of June, the mononucleosis predominated in the blood, the Paul and Bunnell also was still slightly positive. How should one interpret this genital ulceration? Since it was the first symptom and a transitory one it cannot be a trophic ulceration due to the hypogranulocytosis. Was it the point of entrance of the unknown virus of the disease? Another venereal contamination is possible, but there was no reason to suspect it and the wife of the patient remained healthy.

J P S

INFLUENCE OF OESTRONE ON THE LYMPHOID TISSUES OF MALE MICE. L. Dmochowski and E. S. Horning. From Laboratories of the Imperial Cancer Research Fund, Mill Hill, London. J. Path. & Bact. 59 307-312, 1947

The reported higher incidence of tumors in female mice led the present authors to investigate the association of oestrogenic stimulation and neoplasms in male castrate mice. Two strains of male mice were used in 3 groups from each. Group 1 was castrated and then painted with oestrone. Group 2 was painted but not castrated. Group 3 was the control group. The incidence of lymphoid tissue changes in both test groups was much higher than in the control and higher in the castrate than non-castrate. It has not been determined whether or not these lymphoid changes were of a truly malignant nature.

O P J

## LYMPHOMA AND BONE TUMORS

LE TRAITEMENT CHIRURGICAL DE LA MALADIE DE HODGKIN (SURGICAL TREATMENT OF HODGKIN'S DISEASE) Jean Bernard. Paris. Bull. et mém. Soc. méd. d'Hôp. de Paris. 4<sup>e</sup> série, 63<sup>e</sup> année, nos. 23, 24, et 25. 613-616, 1947

Jean Bernard, author with P. Chevallier of an excellent monograph on Hodgkin's disease, reports the results of surgical attempts to treat Hodgkin's disease.

In 6 children, a cervical lymph node which was hypertrophic was removed. In these cases, there were no other adenopathies, and the mediastinum and the spleen were not involved. There was no fever. The diagnosis of Hodgkin's disease was made on histologic examination. Tuberculin reactions were negative, and then were only slight modifications of the white cell counts (neutrophilia and slight eosinophilia).

Two of the 6 children, are still alive and in perfect health, 6 and 8 years after the operation. In the other 4 cases the disease progressed as if no surgical attempt had been made. After 3 to 12 months new adenopathies appeared. The surgical procedure was excision of the hypertrophic lymph node, without

"evidemment ganglionnaire" At this initial period, the clinical diagnosis of Hodgkin's disease is only presumptive before histologic examination. The unnecessary removal of an inflammatory lymph node is preferable to losing precious time awaiting a more characteristic picture.

These observations have a theoretic interest. Hodgkin's disease seems to have a short, localized, initial stage, even if it is a system disease (as the fowl-leucosis has a first, medullary, localized stage). The practical interest is considerable. Since the surgical removal has no detrimental effects on the course of the disease, two successful results in six attempts bring hope in the treatment of this fatal disease.

J P S

**TUMORS OF THE SKELETAL SYSTEM. MEDICAL ASPECTS.** *A B Gutman* From the Department of Medicine, Columbia University College of Physicians and Surgeons and the Presbyterian Hospital, New York, N Y. *Bull New York Acad Med* 23: 512-518, 1947.

The clinical hematologist who is often called upon to assist in the differential diagnosis of bone tumors will find much of interest in this straightforward presentation. The significance of abnormal blood levels of calcium, inorganic phosphate, alkaline phosphatase, acid phosphatase and serum proteins is given in tabular form and briefly explained. Recent advances in medical treatment of bone tumors are cited as examples of current trends but are not discussed.

L E Y

**TUMORS OF THE SKELETAL SYSTEM. PATHOLOGICAL ASPECTS.** *H L Jaffe* From the Hospital for Joint Diseases, New York, N Y. *Bull New York Acad Med* 23: 497-511, 1947.

This concise presentation, like the above, merits attention from those interested in the blood and blood-forming organs. The pathologic and some of the clinical features of the most important bone tumors are clearly described. The author recognizes two general cytologic groups of multiple myeloma, namely, the plasma cell myeloma or plasmacytoma, and a group of more varied cytology dominated by cells larger than plasma cells. The pathognomonic findings in the kidneys in this disease and the presence of amyloid in some cases are stressed. With regard to roentgenographic evidence of multiple myeloma, it is emphasized that when films of the other bones do not show the conventional picture, the calvarium as a rule also fails to show it.

L E Y

## LYMPHOMATOUS DISEASE

**MALIGNANT LYMPHOMA. THE VALUE OF RADICAL SURGERY IN SELECTED CASES.** *C A Hellwig* From the Department of Pathology, St. Francis Hospital, Wichita, Kansas. *Surg Gynec & Obst* 84: 950-958, 1947.

An analysis of the clinical and pathological features of 130 cases of malignant lymphoma is presented. A close correlation existed between the histology of the tumor and the life expectancy of the patient. Sixty-seven cases were subjected to radical surgery and 63 of these survived the operation. Twenty-one of these patients received no other form of therapy and 12 of these survived 5 years or longer, and showed no evidence of disease at the time of the report.

Forty cases received adequate radiation therapy following radical removal of the tumor. Eighteen of these patients survived and 9 were without evidence of disease at the time of the report.

From reports such as this it is apparent that radical excision of localized accessible lymphomatous tumors should be attempted. Hellwig stresses the fact that radiation therapy alone does not produce cure of malignant lymphomatous disease and actually prolongs the life of its victims very little. In contrast, radical excision of the tumor may occasionally result in complete cure, and frequently prolongs life.

J F R

**ASPIRATION BIOPSY OF LYMPH NODES. A CRITICAL REVIEW OF THE RESULTS OF 300 ASPIRATIONS.** *R E Meatherringham and L F Ackerman* From the Department of Pathology, The Ellis Fischel State Cancer Hospital, Columbia, Missouri. *Surg Gynec & Obst* 84: 1071-1076, 1947.

Details of the technic of aspiration biopsy are given and the simplicity of the procedure is emphasized.

In no instance in the 300 aspirations did serious complications result and there were no cases in which fungation from the needle puncture occurred. One hundred and ninety-seven of the 300 aspirations were successful in that either tumor or lymphoid tissue was secured. Of these, 160 showed the presence of metastatic cancer. One hundred and three cases represented technical failure to obtain sufficient tissue but subsequent clinical course proved only 34 of these to be in error in that the patients subsequently developed cancer.

The greatest value of this technic is in the diagnosis of metastatic cancer. It is of much less value in the diagnosis of malignant lymphomatous disease since it usually is necessary to have an entire lymph node for examination in order adequately to establish this diagnosis.

I F R

ROENTGEN THERAPY IN HODGKIN'S DISEASE *T B Merner and K W Stenstrom* From the University of Minnesota Medical School, Minneapolis. *Radiology* 48: 355-368, 1947.

The authors of this article review in some detail the entire subject of Hodgkin's disease from the point of view of history, etiology, clinical diagnosis and differential diagnosis, and pathological distribution, and then summarize the modes of x-ray therapy with regard to technic and results. Their own experiences have led them to formulate a procedure of irradiation which is somewhat different from that of others.

There are 3 principles in this technic. Recurrences of Hodgkin's tissue are more radioresistant than the original areas, hence it is considered advisable to give a relatively heavier dose at the start of therapy. By the same token, the total amount of irradiation is given in as short a time as possible, not exceeding 2 weeks. Furthermore, since the site of recurrence is unpredictable, prophylactic irradiation is not utilized. The authors' technic, correspondingly, consists of intensive irradiation as soon as the diagnosis is made, a full course of treatment being given to each area involved, starting with the adenopathy which is giving the worst symptoms. Each course consists of between 1000 and 2000 tissue roentgens given in not over 14 days. By this method, the authors have, in a series of 185 cases, a 5-year survival rate of 21 per cent, and a 10-year survival of 8 per cent.

This report discusses, in passing, local resection of Hodgkin's tissue with subsequent irradiation, and discards it in favor of heavy irradiation. It also mentions ordinary spray therapy, as well as prolonged spray therapy in small doses, only to prefer heavier intensive irradiation. There is no discussion of therapy with the nitrogen mustards.

S E

## BLOOD COAGULATION, ANTICOAGULANTS AND HEMORRHAGIC DISEASES

A PHOTOMETRIC ANALYSIS OF THE REACTIONS OF BLOOD COAGULATION *J Linn* Department of Zoology, Syracuse University, Syracuse, N. Y. *J. Cell & Comp. Physiol.* 30: 43-77, 1947.

When fibrin is formed during coagulation there is a progressive increase in turbidity. Photometric methods were devised to measure the light scatter and optical density during this process. Nonclottable proteins influenced the optical properties of this system and were believed to enter into the fibrin structure by being trapped in the process of fibrin formation. Evidence was presented to indicate that the clotting of fibrinogen by thrombin occurs in two stages. First the thrombin activates some of the fibrinogen, then these activated molecules react with nonactivated fibrinogen in a progressive polymerization reaction.

O P J

THE USE OF DICUMAROL AS AN ANTICOAGULANT: EXPERIENCE IN 2,307 CASES *E V Allen, E A Hines, Jr., W F Kvale and N W Barker* From the Division of Medicine, Mayo Clinic, Rochester, Minnesota. *Ann. Int. Med.* 27: 371-381, 1947.

Experience over a six year period with dicumarol is reported. Complications due to the use of the drug were limited to a 3.4 per cent incidence of hemorrhage which was considered serious in 1.8 per cent and included 2 cases of fatal bleeding. In a group of 819 patients with either pulmonary emboli or peripheral venous thrombosis there was a 2 per cent incidence of a further vascular complication while under

therapy The one fatal pulmonary embolus occurred after the patient's prothrombin had returned to normal These data show a marked reduction in expected mortality when compared with its incidence without anticoagulant therapy This report further justifies the spirit of general enthusiasm over the use of anticoagulants in thrombo-embolic disease

C A F

THE ORIGIN AND THE PHYSIOLOGY OF HEPARIN THE SPECIFIC THERAPY IN THROMBOSIS *J E Jorpes* The Caroline Institute, Stockholm, Sweden *Ann Int Med* 27 361-370, 1947

This article comprises a concise authoritative review of the origin, chemistry, action and therapeutic applications of heparin The material is dealt with more fully in the author's monograph on Heparin in Thrombosis, 1946 edition

C A F

CONCENTRATED AQUEOUS HEPARIN A NEW FORM OF INTRAMUSCULAR ADMINISTRATION *D Stats and H Neubof* Mt Sinai Hospital, New York *Am J M Sc* 214 159-162, 1947

Concentrated aqueous heparin was used containing 100 mg/cc and injected intramuscularly at 8 to 12 hour intervals in doses of 50 to 180 mg Maximum effect was attained 4 to 6 hours after injection In 2 per cent of injections local reactions were observed consisting of a small nodule or local discomfort This was felt by the authors to be a simple, safe and therapeutically satisfactory method of administration of heparin

Single injections, however, showed considerable variation in duration of action and insufficient data is reported for the reader to judge the predictability of anticoagulant effect on repeated injections

C A F

SOME OBSERVATIONS ON BLEEDING TENDENCY IN THROMBOCYTOPENIC PURPURA *J G Allen, G Bogardus, L O Jacobson, and C L Spurr* University of Chicago Medical School, Chicago, Illinois *Ann Int Med* 27 382-395, 1947

In this very interesting preliminary report the authors question whether the amount of circulating heparin may be increased in thrombocytopenic purpura By titration of heparinized blood against varying concentrations of protamine sulfate, they find that a greater amount of protamine is required to return the clotting time to normal in thrombocytopenic patients than in controls

Toluidine blue (2.5 mg/kilo body weight) was injected intravenously into 6 patients with thrombocytopenia in view of its known antagonism to heparin Four of these patients had leukemia and 2 had idiopathic thrombocytopenic purpura To judge from the illustration presented, the patients experienced dramatic remission in spontaneous purpura Platelet counts and bleeding times did not appear to be affected, and the authors emphasize that bleeding from ulcerated areas may be little improved Certain inconsistencies in the behavior of the patients to treatment along with the as yet limited data make it necessary to withhold any definite opinion as to the therapeutic value of antiheparin drugs in thrombocytopenic purpura

C A F

SUR LA COAGULATION DU PLASMA EXALATÉ PAR LES CULTURES DE *B. PRODIGIOSUS* *P Fredericq* (Liege, Belgique) *Compt rend Soc de biol* 140 1132, 1947

SUR LA COAGULATION DU PLASMA EXALATÉ PAR LES CULTURES D'ACTINOMYCES *P Fredericq* (Liege, Belgique) *Compt rend Soc de biol* 140 1166, 1947

In these two communications, P Fredericq reports the method of cultivating the prodigiosus or the actinomycetes on a sheet of cellophane The cellophane is washed in distilled water, then the solution is filtered through a Jena filter G 5/3 Then the filtrate is precipitated by five volumes of alcohol (94°C) The precipitate is used to make different dilutions Thus an enzyme is isolated the prodigiosicoagulase and the actinomyceticoagulase which are able to clot an oxalated plasma This coagulase is not a thrombin, since a pure solution of fibrinogen is not clotted The presence of prothrombin is necessary (absorption of prothrombin suppresses in the absence of calcium—trypsin-like activity) Both enzymes are as proteolytic as the trypsin itself and the plasma protease (called by Fredericq, tryptase)

J P S

## RADIOACTIVITY AND RADIOACTIVE ISOTOPES

EFFECTOS DE LA ENERGÍA ATÓMICA EN LA SANGRE (THE EFFECTS OF ATOMIC ENERGY ON THE BLOOD) *Sir Lionel Whitby* La Prensa Médica Argentina 34 14, 648, 1947

The deleterious effects upon the blood of atomic energy and high voltage x-ray brings up the necessity for a thorough study of the problem, and of the more practical methods of immunization.

The penetrating and pernicious effects of the radiations are similar to those produced by the gamma rays of radium. There are three such effects (1) those produced by a sudden and intense irradiation, as observed among the victims of atomic bomb explosion, (2) those produced by repeated small, well tolerated doses, as observed in workers with radioactive substances and in investigators of nuclear physics, and (3) the effects secondary to continuous internal irradiation given off by radioactive substances accumulated in the marrow.

The inhabitants of Hiroshima and Nagasaki subjected to intense and penetrating acute irradiation, showed, first, a latent period, then granulocytopenia, which occurred during the first three weeks, three to five weeks later, thrombocytopenia, with hemorrhagic diathesis, made its appearance, and from five to seven weeks after the explosion, cases of aplastic anemia developed. The sternal marrow in these cases, was found aplastic, physiologically if not always anatomically.

Bones absorb radium, thorium, and radioactive calcium and strontium. The bone marrow is subjected to a continuous bombardment of destructive alpha rays, as observed in workers in the manufacture of luminous watches. Aplastic anemia with an aplastic marrow, or with a hyperplastic impotent marrow, develops as a result of this internal irradiation, and the hematologic picture is similar to that observed in the victims of atomic bomb explosion.

R M S

THE MEDICAL USE OF RADIOACTIVE ISOTOPES. I. RADIOACTIVE ISOTOPES IN HEMATOLOGIC DISTURBANCES AND NEOPLASMS *B E Hall and C H Watkins*, From Division of Medicine, Mayo Clinic, Rochester, Minn. Am J M Sc 213 621-628, 1947

Since radiophosphorus is chiefly deposited in the bone and bone marrow, it is understandable why its therapeutic value should be tested for modifying disturbances in the hematopoietic organs. Hall and Watkins review the cases reported in the literature and add some of their own. Radiophosphorus therapy is an effective method of controlling polycythemia vera. Besides the high cost of material another disadvantage is that the occurrence of leukemia may be accelerated.

O P J

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# NEWS AND VIEWS

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The Metropolitan Life Insurance Company, New York, reports that leukemia takes more than 6,000 lives annually in the United States, many of them among children. The death rate reported from leukemia is said to have more than doubled in twenty-five years and is more than five times that of infantile paralysis, about one and one-half times that of measles, scarlet fever, whooping cough and diabetes combined, and almost equal to that of appendicitis.

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The Surgeon General, U. S. Public Health Service, has appointed an Advisory Board whose function is to designate the proper names for the several anti-Rh blood typing serums licensed under the Biologic Law. The desire is to have recommended a system of nomenclature which will be adequate to cover this phase of human genetics and which can be readily adapted to clinical use, medical teaching, and laboratory diagnosis. Members of the Board are William B. Castle, Jr., Professor of Medicine, Harvard Medical School, Maxwell M. Wintrobe, Professor of Medicine and Department Head, School of Medicine, University of Utah, and Laurence H. Snyder, Professor of Medical Genetics and Dean of the Graduate School, University of Oklahoma.

At a Conference on Nomenclature of the Rh factors which was held at Washington, D. C., on October 20, 1947, and continued through the 21st, the Board heard evidence from various persons qualified in this field. It is expected that an official report of the proceedings will shortly be available.

A letter regarding the above conference has been received from Dr. Alexander S. Wiener.

To the Editor

The National Institute of Health recently held a conference on nomenclature of the Rh factors, in order to settle the problem of labeling Rh antisera. The purpose of this letter is to present my own reaction to this meeting.

As far as I could determine, the consensus of those present was that the original designations of the Rh types as  $rh$ ,  $rh'$ ,  $rh''$ ,  $rh'rh''$ ,  $rh_0Rh_1$  (or  $Rh_0'$ ),  $Rh_2$  (or  $Rh_0''$ ) and  $Rh_1Rh_2$ , based on the designation of the three common Rh antisera as anti- $Rh_0$ , anti- $rh'$ , and anti- $rh''$ , be retained as the standard nomenclature. A number of those present suggested that in addition certain alternative designations, using the letters C-D-E, should be added in parentheses when labeling antisera. The present writer maintains that such a duplicate method of designation is entirely unnecessary and will only cause confusion. Experience with the four Landsteiner blood groups has already proved that confusion is avoided only by the use of a single rational and descriptive nomenclature.

At the meeting the present writer had the opportunity to present his original findings on which the standard nomenclature is based, and also to hear the views of other workers in the field. This has finally made clear the mysterious attraction of notations using symbols like  $XYZ_{xyz}$ , or  $Rh_{12345}$ , instead of the symbols  $Rh_0$ ,  $rh'$ ,  $rh''$ ,  $Hr'$  and  $Hr''$  (evidence for  $Hr_0$  has still not been published). Obviously, to many people notations like I, II, III, IV, are much easier to remember than O, A, B, and AB. In the former case one has but to learn that there are four blood groups. To remember O, A, B, and AB on the other



hand one is compelled to learn that there are two substances A and B, in the blood which form four combinations. This additional effort is quickly repaid, however, in the case of laboratory workers who must be familiar with Landsteiner's rule concerning the reciprocal relationship of agglutinogens and agglutinins, and also for medicolegal experts, to whom, for example, the statement that parents of groups O and AB can only have children of groups A and B is much more intelligible than  $I \times IV$  can only yield II and III. Similarly, it seems easier to learn that there are three factors Rh<sub>1</sub>, Rh<sub>2</sub>, Rh<sub>3</sub> or XYZ than that there are three factors, Rh<sub>0</sub>, Rh' and Rh". Actually, by taking a little extra effort to understand the original nomenclature, one learns at the same time of the special serologic, genetic and clinical position of factor Rh<sub>0</sub> and that rh' and rh" are related very much as A and B are to each other. Thus, by applying what one already knows of the four blood groups, one *immediately* knows the eight Rh types, while those using designations like XYZ must start from the beginning and perhaps never really grasp the subject. It is easy to see, for example, why the mating rh  $\times$  Rh<sub>1</sub> Rh<sub>2</sub> yields almost 50 per cent type Rh<sub>1</sub> and 50 per cent type Rh<sub>2</sub> children with only rare children of types rh' and rh" while this is completely unintelligible when written as Neg  $\times$  Rh<sub>123</sub> or xyz or XYZ.

In addition, the symbol Rh has an important descriptive significance, indicating the origin of our knowledge of this antigen through the study by Landsteiner and Wiener of immune serum prepared by immunizing experimental animals with the blood of rhesus monkeys, which antisera, these workers found, also agglutinate the bloods of about 85 per cent of Caucasian individuals. It was because the first antisera were prepared with the blood of rhesus monkeys that the symbol Rh (the first two letters of the name Rhesus) was chosen to designate the antigen, while anti-Rh was the natural name for the corresponding antiserum. When, with the aid of antisera of human origin, it was later discovered that there are other related antigens in human blood, it was only natural that names be selected for these antigens which showed their relationship to the Rh<sub>0</sub> antigen originally discovered by Landsteiner and Wiener, but at the same time indicated their characteristic differences.

One final point which everyone seems to have overlooked is that as early as 1942 when only Rh<sub>0</sub> and rh' had been found by me, I considered the possibility that these factors might be transmitted by genes that were linked, independent or allelic (WIENER, A. S. Blood Groups and Transfusion, ed. 3, 1943, p. 254). When statistical analysis of the available data proved that the hypotheses of linked and independent genes were incorrect, we explained the hereditary transmission of the four Rh types known at that time by postulating the existence of four allelic genes. When later rh" was found, it hardly seemed necessary to repeat the evidence against linkage and independent assortment, and so the theory was merely extended to include six standard allelic genes, to which British workers added the seventh and rarest gene R<sup>s</sup>. Thus, Fisher's theory besides being wrong, is not original since it had been considered and discarded by me more than two years before he proposed it. Incidentally, based on the theory of multiple allelic genes, the existence of type rh'rh" and its frequency of only about 1 in 10,000 was predicted by me and later confirmed. Also the existence of factor rh'" was predicted by me, only to be confirmed by workers in England.

In conclusion, it should be apparent from the foregoing that a recent report published in this journal (Blood 2: 204-27, March, 1947), advocating the adoption of Fisher's notations in order to "clarify the nomenclature problem" is based on incorrect and incomplete information. Actually there is no nomenclature problem except the one that would be created by the unnecessary introduction of duplicate or triplicate systems of notations. Therefore, it is hoped that the publication of this letter will serve to correct any damage caused by the misleading report referred to above.

Alexander S. Wiener

Office of the Chief Medical Examiner, New York, New York.

The report, referred to above, published in this journal was entirely reportorial and represented the consensus of the workers who met at Dallas and Mexico City in November 1946 to discuss problems relating to the Rh factor. The report itself was, we believe, clearly written and therefore not 'misleading'. Dr. Wiener probably objects to the conclusions of the group which met, discussed and arrived at a decision on a question of nomenclature. This decision happens to be at variance

with Dr Wiener's concepts. It will be of interest to obtain the reaction of other readers to Dr Wiener's comments.

*Editor*

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The following items have been received from Dr José Oria, São Paulo, Brazil. The Department of Histology and Embryology of the Medical School of the University of São Paulo, Brazil has been doing histologic research, both human and comparative, of blood and hemopoietic organs for about twenty years. In charge of this work is Assistant Professor Dr José Oria.

Several general and individual contributions to Hematology have had their origin from this department. Thus, the clinical hematologic service of the University is now being carried on in the Central Laboratory of the Clinical Hospital (of the University), under the direction of Assistant Professor of Internal Medicine, Dr Michael Jamra. This service conducts the hematologic examinations of the entire hospital.

Clinical and cytohematologic studies have been published from or are being carried on in both departments. Among others should be mentioned the most recent on megakaryocytes, mast-cells, plasmacytes, artificial segmentation of the blood elements in oxalated milieu (Dr José Oria), on hypersegmented neutrophils (Dr Oria and Dr S. Yoneda), multipolar mitoses in megakaryocytes, drepanocytic anemia, hemophilia, metabolism in pernicious anemia. The karyologic curve of bone marrow in vivo under certain conditions is being studied by Dr Fernando T. Mendes. Dr Jamra and Dr Mendes (of the Clinical Hospital of São Paulo) are in constant contact with the Department of Histology and Embryology of São Paulo, in order to coordinate these services.

The Section of Cellular Physiology of the Department of Histology and Embryology (Dr Piero Manginelli, former Research Associate with Dr R. Chambers) is initiating cytologic investigations in blood.

Dr Lucio Carvalho Lima, voluntary assistant in this Department, is now at work in Dr M. M. Wintrobe's laboratory at the University of Utah.

Dr Carlos A. Lacaz and co-workers have written on the Rh factor in São Paulo. Their work is being performed in the Department of Microbiology and in the Clinical Hospital of the same University. One of Dr Lacaz's co-workers is Dr Humberto Costa Ferreira, who worked recently with Dr Nathan Rosenthal in New York.

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The eighth annual meeting of the Italian Society of Hematology was held in Pavia, June 29 - 30, 1947. The French and Swiss Society of Hematology were represented.

The presidential address was given by Prof. Di Guglielmo of Naples. Drs. Conte-Marotta and Di Guglielmo Jr. spoke on 'Urinary Factors in Leukemia,' Dr Fieschi spoke on 'Marrow Cultures in Leukemia,' Dr Storti on 'Leukemia as Neoplasm,' Dr Baserga on 'Recent Advances in Pathogenesis and Therapy of Leukemia,' and Dr Morganti on 'The Rh Factor.'

The following letter, dated July 20, 1947, from Dr Paul Chevallier of Paris, addressed to "Mr President of the American Society of Hematology" has been received at the editorial office. It was translated by Dr H Tagnon. At present, no American Society of Hematology has been constituted.

Mr President

I have the honor of informing you that at the Italian Hematologic Congress June 29-30, 1947 it was decided to create an Academy of Hematology. The word Academy was chosen for the time being rather than Society or Association, to indicate that the number of members is limited and that, in agreement with the meaning of the French word *academique* no other condition should be considered except that of hematologic science.

For the time being, the Academy has been qualified as European, this because no representative from another continent was present. The founders have the strongest desire that it be International.

It was also decided that in the present world contingency, the general secretary (permanent) could not be otherwise than a Swiss citizen with perfect neutrality. Sr Undritz was elected but his definite acceptance is conditioned on the decision of his superiors.

I was elected President and this is why I am writing you for this embryo of an Academy.

In order to keep the Academy young, the active members, with the same privileges, have been divided into *seniors* (above 65 or 70 years, or elected for more than the last 10 years), without limitation in number, and *juniors*, whose number has been provisionally limited to 100 (for Europe alone).

In addition there will be members who will participate in the scientific proceedings but not in the administration. These are corresponding members and *Membres agrees*, appointed for 5 years and chosen among those of the young who show definite promise but as yet have not attained a definitive status as hematologists.

In those countries having a National Hematologic Society, membership will depend on nomination by the National Society.

The Academy will hold annual meetings, in a different country, lasting two days, and will study various problems submitted. One meeting will be devoted to presentation of scientific and clinical material by the hematologists of the region where the meeting is held.

It is projected to have the first meeting (study of constitution, by-laws, etc.) in Paris, in May 1948.

In the meantime, the provisional Bureau (Paul Chevallier, di Guglielmo, Lambin, Undritz) is commissioned to choose a certain number of known hematologists to form the nucleus of the Academy.

We have the great hope that you will join us and bring us your admirable vitality. On you depends the International rather than European character of the Academy.

If, as we hope, you agree, you will kindly send us the names of people whom your Society thinks should be Founder members, and a list of the candidates for election to vacant seats.

We will also have to discuss with your representative the problem of the total number of junior members of the *International Academy*, the junior members from Europe being limited to one hundred.

I beg you to receive, Mr President, the expression of my very high consideration.

Paul Chevallier

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### *International Society of Hematology*

Much progress is being made in the development of this Society, which evolved from the highly successful Rh and Hematology Congress held in Dallas and Mexico City in November, 1946. The officers are: President, Dr Joseph M Hill, Dallas, Vice-President, Dr Eduardo Uribe Guerola, Mexico City, Secretary, Dr Sol Haberman, Dallas, Treasurer, Dr Stanbury, Toronto.

Meetings are to be held bi-annually, probably during one of the summer months, so as to coincide with vacation plans and travel. The first of these meetings will

be held at the Hotel Statler, Buffalo, during August 24-27 inclusive Tentative plans indicate that these four days will be divided as follows one day, red cell problems, one day, immuno-hematology, one-half day, white cell problems, one-half day, coagulation problems, one-half day business, one-half day entertainment

The following Committees have been appointed

*Program Committee*

Witebsky, Ernest, Chairman	Buffalo General Hospital, Buffalo, New York
Diamond, Louis K	The Blood Grouping Laboratory, 300 Longwood Ave, Boston, Mass
Whitby, Sir Lionel	Regius Prof of Physics, Department of Medicine, University of Cambridge, London, England
Moore, Carl	600 S Kings Highway, Washington University School of Medicine, St Louis, Missouri
Guerola, Uribe Eduardo	Ponciano Arriaga 26, Mexico, D F
Seegers, Walter	Wayne University, Detroit, Michigan
Snyder, L H	Oklahoma University, Dean of Graduate School, Norman, Oklahoma

*Constitutional Committee*

Davidsohn, Israel, Chairman	Mt Sinai Hospital, Department of Pathology, California Ave and 15th Place, Chicago 8, Illinois
Gutierrez Villegas, Luis	Plaza de la Republica 52, Mexico, D F
Robb Smith, A H T	University of Oxford, Director of Pathology, Radcliffe Infirmary, Oxford, England
Tocantins, L M	Jefferson Medical College and Hospital, Philadelphia, Pennsylvania
Ross, Joseph	Evans Memorial Laboratory, Memorial Hospital, Boston, Mass

*Membership Committee*

Dameshek, William, Chairman	25 Bennet St, Boston, Mass
Levine, Philip	Ortho Research Foundation, Raritan, N J
Jones, O P	University of Buffalo College of Medicine, Buffalo, N Y
Custer, R Phillip	University of Pennsylvania, Medical School, Dept of Pathology, Philadelphia, Penn
Kracke, Roy R	University of Alabama Medical School, Dean, Birmingham, Alabama

*Foreign Countries*

Race, Robert R	Lister Institute, Chelsea Bridge Rd, London, S W 1, England
Hirsfeld, Ludwig	Institute of Medical and Microbiological Science, Wracław, Poland
Guzman, Ignacio González	University of Mexico, College of Medicine, Mexico, D F
Cruz, Walter	Instituto Oswaldo Cruz, Caixa Postal 926, Rio de Janeiro, Brazil
Pavlovsky, Alfredo	Ancherena 1710, Buenos Aires, Argentina
Waugh, Theodore	McGill University, Montreal, Quebec
Broman, Berger	Royal Caroline Medical School, Stockholm, Sweden
Bessis, M	Laboratoire de Recherches, Du Centre National de Transfusion Sanguine, 53 Boulevard Diderot, Paris, France
Das Gupta, C R	Hematology Department, Calcutta School of Tropical Medicine, Calcutta, India
Sandoval S, Luis	Instituto de Histologia de la Universidad de Concepcion, Santiago, Chile
Sirivejkul, Rod	Siamese Medical Department, The Royal Siamese Embassy, Washington, D C
Rohr, Karl	Medizinischen Universitätsklinik, Zürich, Switzerland

Chediak, Moises

Laboratorios Chediak, 23 No 654 Esq A Banos Vedado, Habana,  
Cuba

Di Guglielmo, G

Director of Medical Clinics, University of Naples, Policlinico,  
Napoli, Italy

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### Blood Bank Institute

A Blood Bank Institute was held in November 1947 at Dallas, Texas in conjunction with the William Buchanan Blood Center. Eighty-three representatives of blood banks from various parts of the country attended and participated in the program. At the conclusion of the program an American Association of Blood Banks was formed. Details regarding both the institute and the new Association will be published in a forthcoming issue of this Journal.

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### To the Editor

In two papers<sup>1, 2</sup> appearing in recent issues of *Blood*, reference has been made to lysolecithinase as the enzyme which converts lecithin to lysolecithin.

This terminology is, of course, incorrect: an enzyme is not named from the product it forms. Following the usage of Belfanti<sup>3</sup> the enzyme in question is commonly referred to as lecithinase A. It would be unfortunate if the incorrect term were generally adopted by hematologists.

H. Bruce Collier

<sup>1</sup> SINGER, T. P., I. SILBERBACH, AND S. SCHWARTZ. *Blood* (Special Issue) 1: 88-97, July 1947.

<sup>2</sup> HIRSCHDOECK, J. S. *Blood* 2: 578-591, 1947.

<sup>3</sup> BELFANTI, S., A. CONTARDI, AND A. ERCOLI. *Ergeb. Enzymforschung* 5: 213-232, 1936.

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# BOOK REVIEW

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*The Coagulation of the Blood Investigations on a New Clotting Factor* BY PAUL A. OWREN Oslo, J. Christian Gundersen, 1947

In this book, which was also published as a supplement to *Acta Medica Scandinavica* in 1947, Owren describes an unusual case of a hemorrhagic diathesis in a woman with increased coagulation time in which, at first sight, prothrombin appeared to be at fault. All the other elements of the blood coagulation reaction were apparently normal, quantitatively and qualitatively. Furthermore, laboratory findings revealed no apparent cause for the hemorrhagic tendency in this patient other than a severe hypoprothrombinemia as determined by the Quick method. Subsequent investigations by the author demonstrated that addition of small amounts of prothrombin free plasma produced a great decrease in the prothrombin time of the patient and consequently an apparent increase in the prothrombin concentration as determined by Quick's method. Since Owren found that fibrinogen, prothrombin, thrombokinase, and calcium were all present in normal amounts he concluded that some factor other than those usually associated with coagulation was at fault. By precipitation with weak acids, dialysis, and salting out methods, Owren was able to prepare a material which apparently was lacking in his patient and which he terms Fraction 5. This factor he claims is necessary for normal coagulation of plasma and is found in normal plasma after the removal of calcium, prothrombin, fibrinogen and by means of Chamberland filtration, thrombokinase. The addition of Fraction 5 to the customary Quick setup results, according to Owren's data, in a markedly decreased prothrombin time. Owren assumes that Fraction 5 requires either prothrombin or a portion of the prothrombin complex to form a second new substance, Fraction 6, in the presence of calcium or thrombokinase. Fraction 6 which has as yet not been isolated, reacts with prothrombin in the presence of calcium to form thrombin which in turn produces the fibrin clot from fibrinogen. Owren has suggested the term proprothrombinase for Fraction 5 and prothrombinase for Fraction 6. He advances the view that Fraction 6 is a proteolytic enzyme capable of converting prothrombin to thrombin. He replaces the older terminology of Bordet and Nolf by a new term, cytokinase, which replaces the old term cytozyme and thrombozyme. Owren's theory of blood coagulation, then, may be schematically described as follows:

1. Factor V Prothrombin (?) Cytokinase Ca Prothrombinase
2. Prothrombin Prothrombinase Ca Thrombin
3. Fibrinogen Thrombin Fibrin

Owren's theory has been republished in part in *Lancet* and has been editorialized by that journal.

The acceptance of two new members of the coagulation family is a serious development requiring careful consideration on the part of all investigators in the field and undoubtedly much investigation will be stimulated as a result of Owren's work. There is, however, a considerable danger involved in accepting this new concept at the present time. The methods of preparation of Fraction 5 are, in a great many ways, similar to those used by Taylor and associates for the preparation of globulin substance or antihemophilic globulin. Plasmas may be prepared free from formed elements and from prothrombin and fibrinogen, but which still contain significant amounts of globulin substance by precipitation with weak acids or on dialysis. There is also a similarity between Fraction 5 and the so-called activating globulin of Seegers. Since the greatest difficulty of the study of blood has been the failure of investigators to relate the various preparations they have made with preparations of others having similar chemical properties and physiologic functions, it would seem highly important that Owren's Fraction 5 be fully investigated definitively before it can be accepted as a new factor. Fraction 6 which has not yet been isolated in many of its properties, resembles the proteolytic enzyme described by Tagnon and his associates and which we now know can be prepared in free form by the fractionation procedures of Cohn. Such proteolytic enzymes are an important consideration in the theory of Ferguson. Furthermore, there is some evidence that the postulation of a substance such as Fraction 6 is unnecessary when one considers the coagulation

properties of freshly prepared and old serum. Owren's work is a challenge for further investigation in an attempt to clarify the coagulation situation. No theory at present competently explains all the facts.

In addition to the presentation of his new hypothesis, Owren has reinvestigated the kinetics of the coagulation reaction from many angles and has re-examined the physico-chemical properties of the classic components of the coagulation reaction. In chapter 8, he attempts to bring his present investigation into relation with the older theory of blood coagulation.

Unfortunately, a great deal of work in the United States since 1941 was not available to the author, and as expressed by Owren, only Danish and scattered British and American articles have been available for reference. No student of the blood coagulation problem should fail to review Owren's work in an attempt to relate the new components to the classic theory.

F. H. L. Taylor, Boston

# CONGENITAL HEMOLYTIC JAUNDICE THE PATHOGENESIS OF THE "HEMOLYTIC CRISIS"

By PAUL A. OWREN, M.D.

THE MOST serious and dramatic complication in congenital hemolytic jaundice is the occurrence of a hemolytic crisis. This manifests itself by a sudden catastrophic fall in the hemoglobin and red cell count which in the course of a few days can produce a critical, sometimes fatal condition in the patient.

The immediate cause of these acute crises is unknown, but most hematologists seem to be agreed, as far as the pathogenesis is concerned, that the acute anemia arises because of a sudden greatly increased destruction of the red cells, a view which is expressed in most of the standard text books and papers on this subject (Meulengracht 1918, Lord Dawson of Penn 1931, Heilmeyer 1935, Schulten 1939, Vaughan 1936, Kracke 1941, Whitby and Britton 1946, Wintrobe 1946, and others). The terms 'hemolytic crisis,' 'hemoclastic crisis' and 'crise de deglobulisation' express this view.

I shall refer later to investigations which show that this generally accepted view may be erroneous. It will be shown that the anemic crises are not due to increased hemolysis, but to a sudden cessation in formation of the new red cells because of an acute aplastic crisis in the blood forming tissue of the bone marrow.

## MATERIAL

The investigation covers crises in 6 cases of congenital hemolytic jaundice. The first 4 cases, which occurred in the members of the same family, were observed in 1942. The following members of this family became ill within a few days of each other: (1) An 8 year old girl, (2) her grandfather of 59, (3) her father aged 40, (4) her father's brother aged 38. The fifth case was a man of 22 whose family connections are shown in figure 1. This family tree is of special historical and clinical interest for the following reasons: 3 members of the second generation had anemic crises in 1890, 10 years before Minkowski's description of congenital hemolytic jaundice. This incident was described by Koren in 1891 as '3 cases of acute pernicious anaemia in the same family,' and Torup (1891) suggested that the anemia was produced by an acute carbon monoxide poisoning. This erroneous explanation resulted in the subsequent view that carbon monoxide poisoning can produce marked anemia. The true reason for the incident was given by Holst in 1917. The sixth case in a woman of 20, belonged to a third family.

In all 6 cases the disease showed complete accordance with the criteria for hemolytic jaundice, which was first described by Minkowski in 1900 and Chauffard in 1907. The patients presented the following characteristics: jaundice without bilirubin in the urine, anemia, splenomegaly, increased fragility of the red cells.



to hypotonic saline, increased number of reticulocytes in the blood, spherocytosis and familial incidence of the disease

All patients were examined several times after complete recovery from the crisis, two of them also before onset of the crisis. Representative findings in the peripheral blood are given in table 1

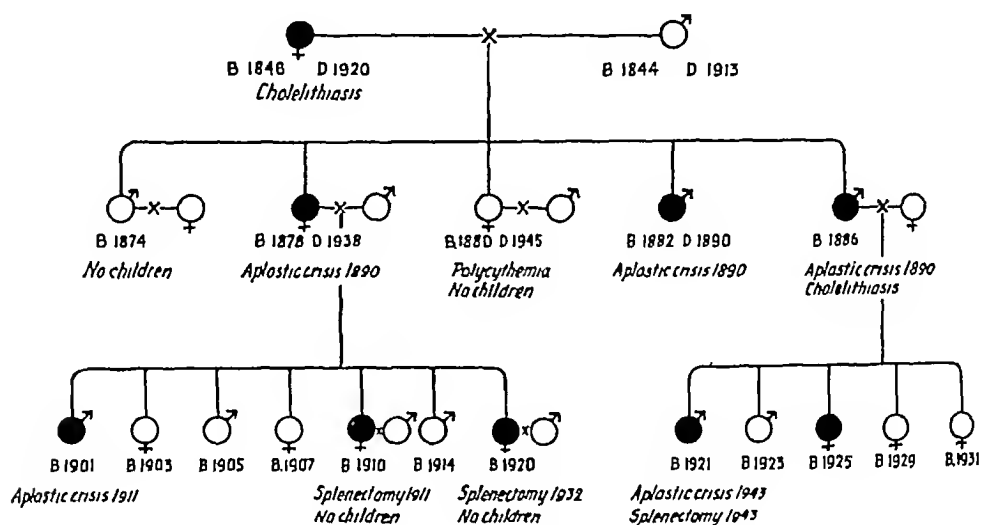


FIG 1 FAMILY TREE OF PATIENT 5, MALE 22 YEARS

Aplastic crisis and splenoctomy 1943. Three members of the second generation had crises in 1890

TABLE 1—Blood values before and after recovery from crises in 6 cases of congenital hemolytic jaundice

Cases no	Sex	Age	Hgb	RBC $\times 10^6$	WBC	Thromboc	Retics	Fragility	Mean diam	Icterus index	Date of examination
			%				%		$\mu$		
1	♀	8	86	4.22	5,300	260,000	5.2	0.64 0.40	6.5	10	4 months after crisis
2	♂	59	62	2.55	4,600	175,000	14.0	0.56 0.34	6.8	16	4 months after crisis
3	♂	40	78	3.53	7,100	310,000	10.0	0.70 0.50	6.7	14	2 months after crisis
4	♂	38	98	4.58	5,600	270,000	4.7	0.68 0.44	6.8	20	8 days before crisis
5	♂	22	83	4.06	5,200	219,000	7.3	0.72 0.42	7.1	16	3 months after crisis
6	♀	20	80	3.82	4,700	299,000	8.0	0.74 0.34	6.6	12	1 year before crisis

#### CLINICAL AND HEMATOLOGIC FINDINGS DURING THE COURSE OF THE CRISES

In all 6 cases the crises began quite suddenly with a rise in temperature, cases 2 and 4 having rigors, and case 1 abdominal pain and vomiting. The temperature swung irregularly between 38° C and 39.5° C, lasted about ten days and returned to normal at the same time as the recovery of the blood picture began. Jaundice

decreased in all cases with the development of the anemia (cf figs 2, 10, 12), and the size of the spleen remained unchanged during the course of the crisis, as far as its size can be determined clinically. These points are emphasized, as the literature states that the jaundice as well as the size of the spleen increase during the crisis.

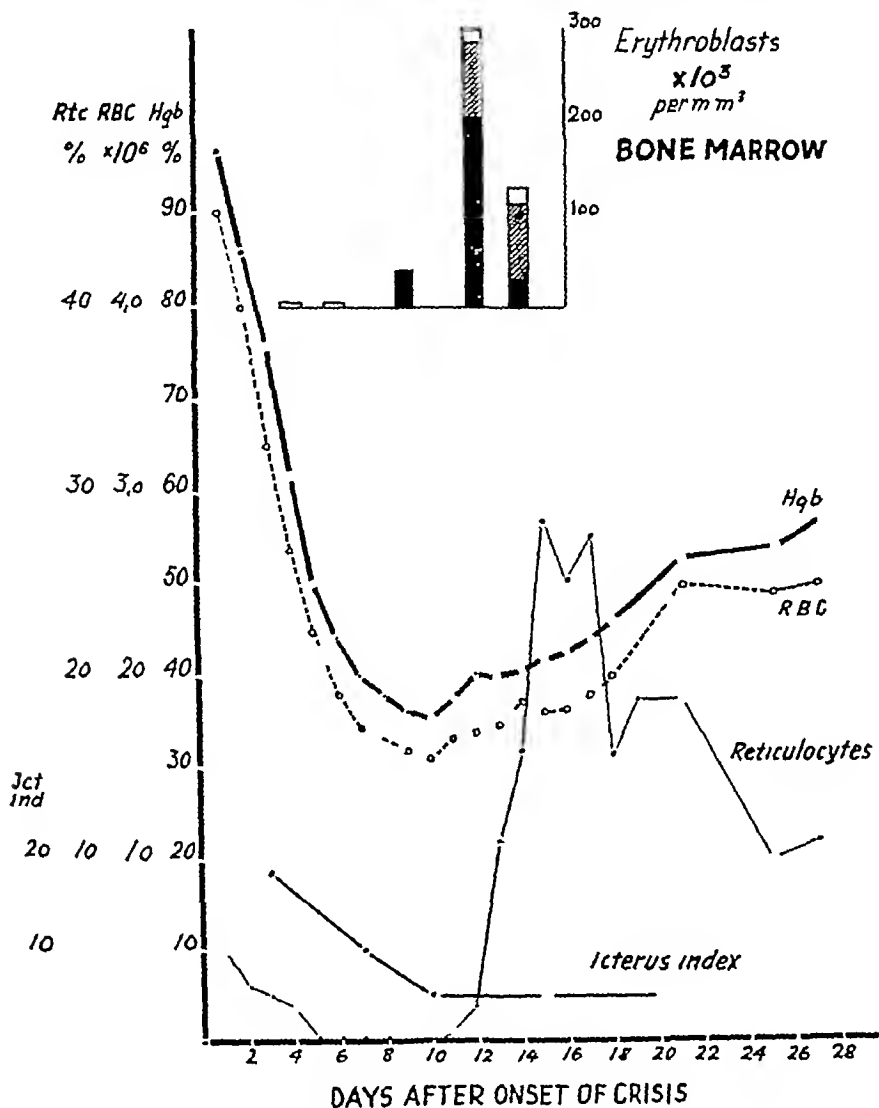


FIG. 2. CASE 4. MALE 38 YEARS. BLOOD AND MARROW FINDINGS.

Black, basophilic erythroblasts, slantlines, polychromatic erythroblasts, open, orthochromatic erythroblasts.

#### THE ERYTHROPOIETIC FUNCTION

The typical hematologic findings during the course of a crisis are given in figure 2, which gives the blood and marrow findings in case 4, a man aged 38.

Eight days before he became ill, this patient went to the hospital with his father, case 2, and a full clinical and hematologic examination was made at that time. He presented all the characteristic signs and symptoms of hemolytic jaundice (cf table 1), and was told to report for admission to the hospital immediately if he felt ill. Eight days later he felt cold and chilly, and his temperature started to rise,

the following day he reported to the medical ward. In this way we had a unique chance to collect observations just before the crisis as well as during its development. As a rule patients with "hemolytic" crises do not come under observation before the anemia is fully developed, as it is the anemic symptoms which bring the patient to the doctor. This fact seems to be the reason that the first phase of the crisis, the onset of anemia, has not been fully reported until now.

It is evident from figure 2 that the anemic phase is characterized by the following findings: the hemoglobin and red cell count fall rapidly, and in the course of about six days are reduced to about half of the original value. The color of the serum

TABLE 2—Case 4 Number of nucleated cells in bone marrow during crisis

	Eight days before crisis	Days after onset of crisis				
		4	6	9	12	14
Erythroblasts, %	53.0	4.8	4.2	29.0	81.0	60.0
Leukoblasts and leucocytes, %	47.0	95.2	95.8	71.0	19.0	40.0
Nucleated cells per cu. mm.	270,000	90,000	70,000	125,000	380,000	220,000
Erythroblasts per cu. mm.	143,000	4,300	2,900	36,000	308,000	133,000
Leukoblasts and leucocytes per cu. mm.	127,000	85,700	67,100	89,000	72,000	87,000

TABLE 3—Case 4 Differential counts of nucleated red cells in bone marrow

	Eight days before crisis	Days after onset of crisis				
		4	6	9	12	14
Erythropoiesis per cent	53	4.8	4.2	28.8	81.0	60.5
Proerythroblasts	1.8	0.1	0.1	9.7	2.0	1.2
Macroblasts	12.5	0.5	0.4	14.5	55.1	13.2
Polychromatic normoblasts	23.1	0.4	0.6	2.0	19.0	39.1
Orthochromatic normoblasts	4.5	1.0	0.5	0.5	3.9	5.4
Pyknotic normoblasts	—	2.8	2.6	1.2	—	—
Mitotic normoblasts	1.1	—	—	0.9	1.0	1.2

becomes normal again, and the reticulocytes disappear completely from the peripheral blood.

The reticulocyte disappearance points to the fact that the anemic phase is accompanied by a complete cessation of the erythropoietic function. This is fully confirmed by examination of the bone marrow. The upper part of figure 2 illustrates the number of erythroblasts per cu. mm. bone marrow. In the anemic phase there is almost complete aplasia of the erythropoietic tissue as is otherwise seen only occasionally in aplastic anemia. Remnants only of erythropoietic tissue are found in this period in the form of a very few stunted normoblasts, and scattered large characteristic immature cells. At the first sign of recovery we see active formation from these large cells of proerythroblasts (pronormoblasts) with subsequent development to macroblasts (basophilic normoblasts) and later polychromatic and orthochromatic normoblasts which in the course of a few days increase so that

they completely dominate the bone marrow picture in an excessively active proliferation (cf fig 2) This regeneration is immediately followed by a reticulocyte crisis in the peripheral blood, up to 30 per cent, with a subsequent quick recovery of the blood picture

Table 2 illustrates the relative rates of this regeneration The number of erythroblasts which, during the initial phase of the crisis, are reduced to about 3000 per cu mm bone marrow or 4-5 per cent of the total amount of nucleated cells, increase rapidly to about 300,000 per cu mm or to 80 per cent of the nucleated cells

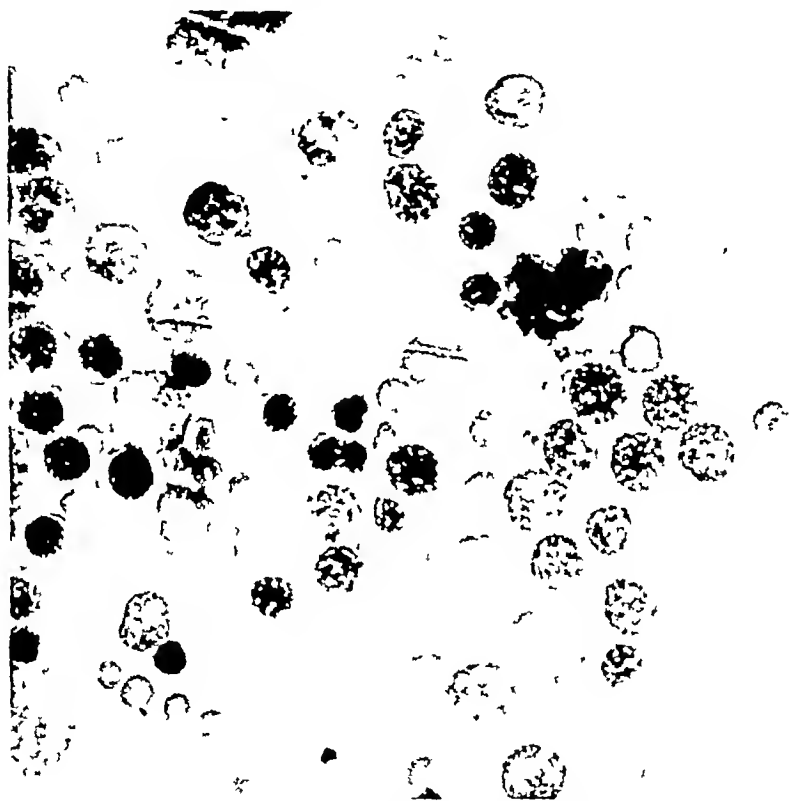


FIG 3 CASE 4 BONE MARROW 8 DAYS BEFORE CRISIS THE ERYTHROPOIESIS DOMINATES THE PICTURE  $\times 600$

The qualitative changes during this regeneration as they appear in the differential count are given in table 3 From this it is clear that the course of regeneration goes through the normal stages of proerythroblasts and macroblasts to the hemoglobin containing polychromatic and orthochromatic normoblasts

The photomicrographs verify this development Figure 3, which shows the bone marrow in this patient eight days before crisis, shows a typical and uncomplicated picture of hemolytic jaundice, the erythropoiesis dominating the picture and the more mature normoblast stages forming the most striking component On the fourth day of the crisis (fig 4) the picture is completely changed the erythroblasts have disappeared and the marrow consists almost exclusively of leukopoietic tissue, with myelocytes and promyelocytes predominating On the sixth day the picture is similar (fig 5)

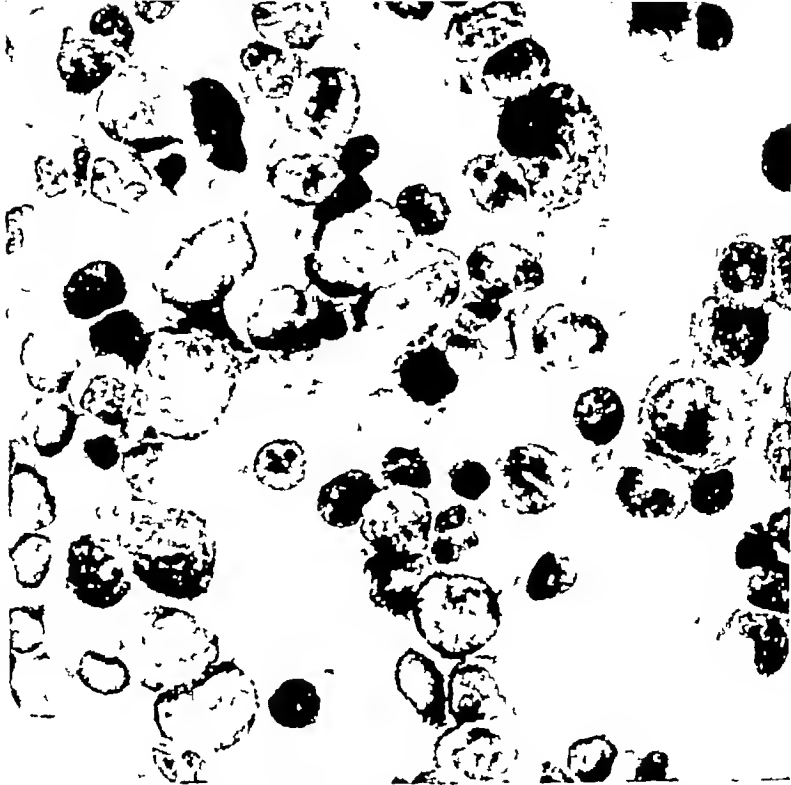


FIG 4 CASE 4 BONE MARROW PICTURE ON THE FOURTH DAY AFTER ONSET OF CRISIS

The erythroblasts have almost completely disappeared and the marrow consists almost exclusively of leukopoietic tissue  $\times 600$

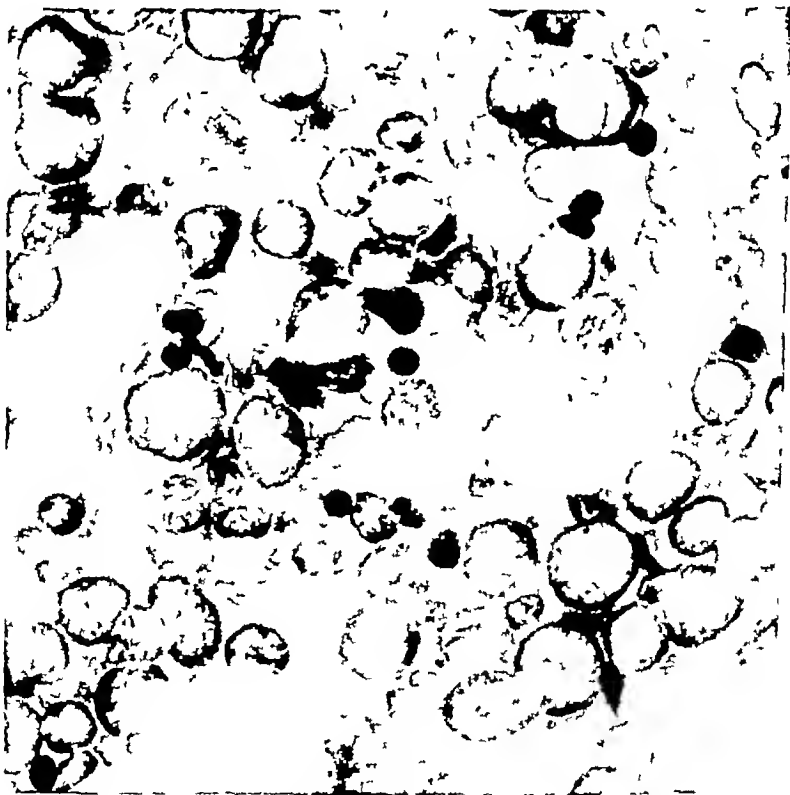


FIG 5 CASE 4 SIXTH DAY OF CRISIS

The picture is similar to fig 4  $\times 600$

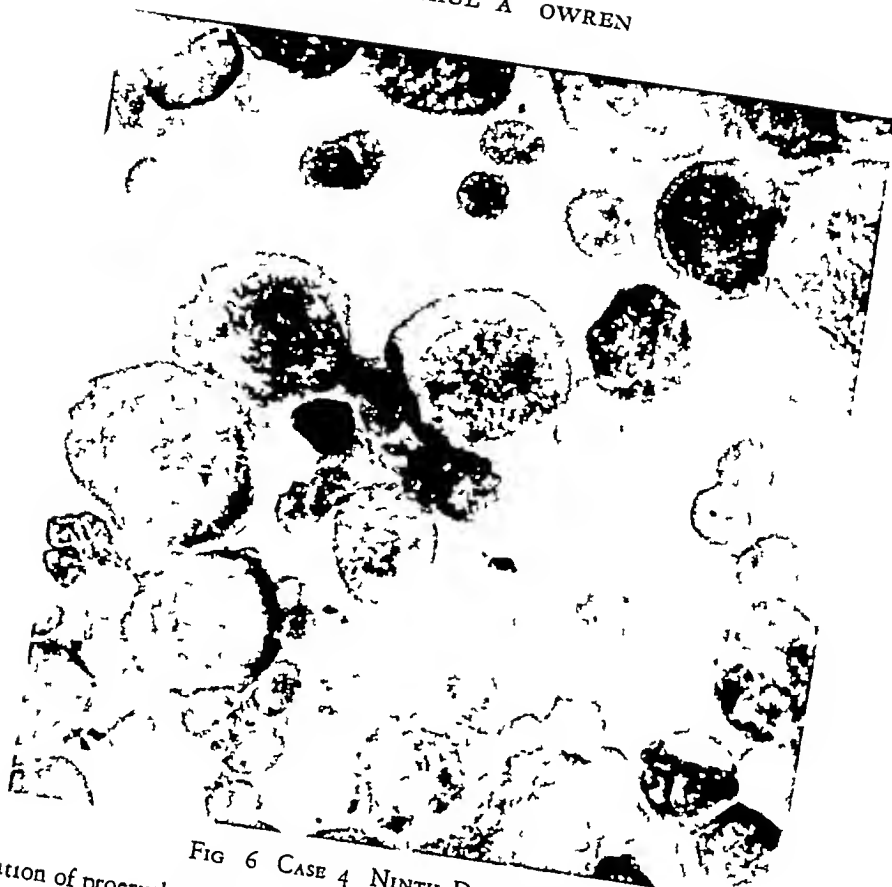


FIG 6 CASE 4 NINTH DAY OF CRISIS  
The regeneration of proerythroblasts has started  $\times 1000$

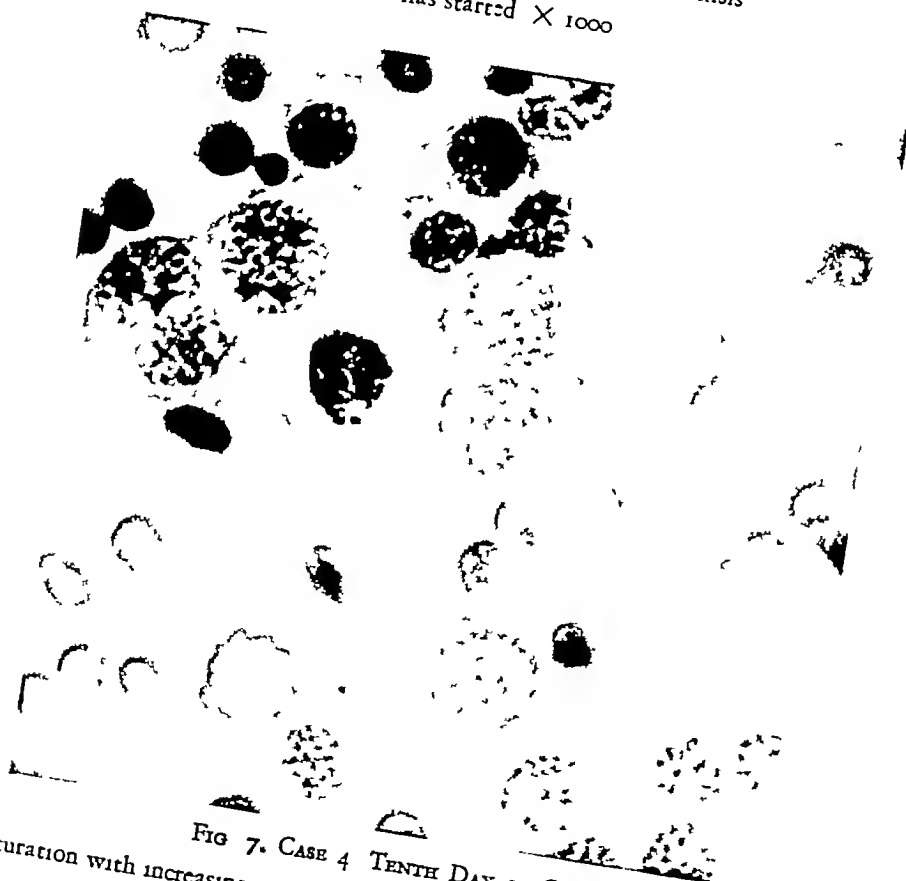


FIG 7. CASE 4 TENTH DAY OF CRISIS  
Further maturation with increasing macroblasts  $\times 1000$

Which cell in this marrow is the origin of regeneration of the red cells? As mentioned, preparations from this period show single large cells, 35 to 40 $\mu$  in diameter and with a basophilic protoplasm and leptochromatic nuclei. From these cells one can follow a uniform development to proerythroblasts (fig 6) from the ninth day of the crisis, and (fig 7) from the 10th day. At this time we find a regeneration of proerythroblasts in great quantities, together with a number of macroblasts (basophilic normoblasts), but not more mature forms. There are still no reticulocytes in the peripheral blood. Figure 8, taken two days later, shows an enormous increase in normoblasts and a great diminution in the proerythroblasts. On the

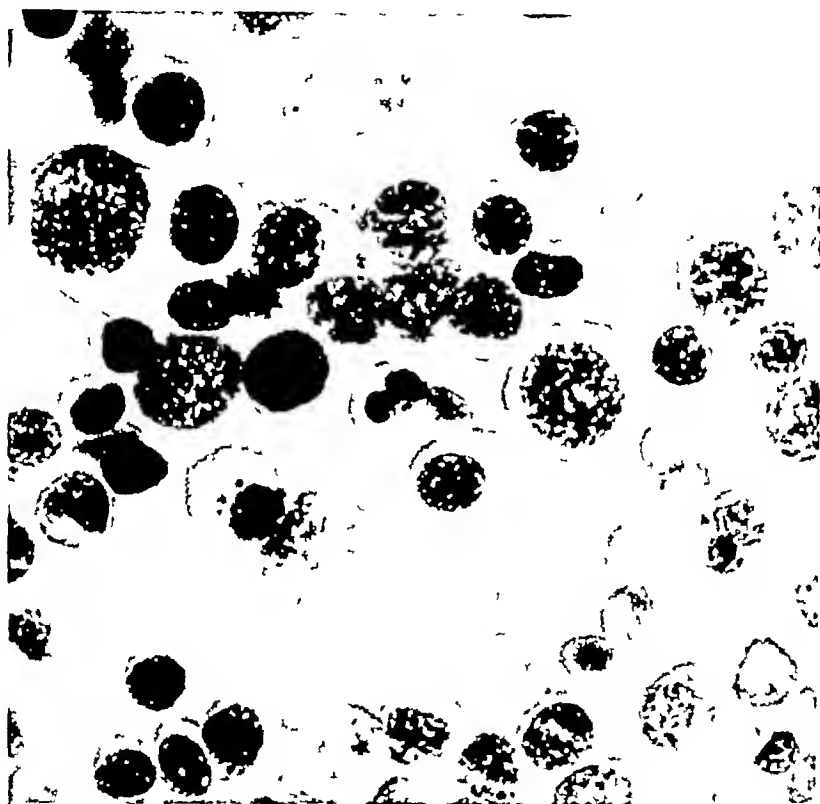


FIG 8 CASE 4 TWELFTH DAY OF CRISIS

Large increase of normoblasts and decreasing number of proerythroblasts  $\times 1000$

fourteenth day (fig 9), we find large nests of polychromatic normoblasts. At this time the reticulocytes in the peripheral blood were increased to 30 per cent.

It is apparent from these findings that during the development of anemia the erythropoietic tissue of the bone marrow is in a temporary state of aplasia. The spontaneous recovery shows that the erythron has a quite fantastic ability to regenerate.

Figure 10 shows the course of the crisis in patient number 6, a woman aged 20. The color of the serum diminished and the jaundice disappeared. Reticulocytes disappeared from both peripheral and sternal blood, and recovery was heralded by a reticulocyte crisis. The sternal marrow was examined daily in this case, and showed similar changes as in the previous patient. The differential counts of

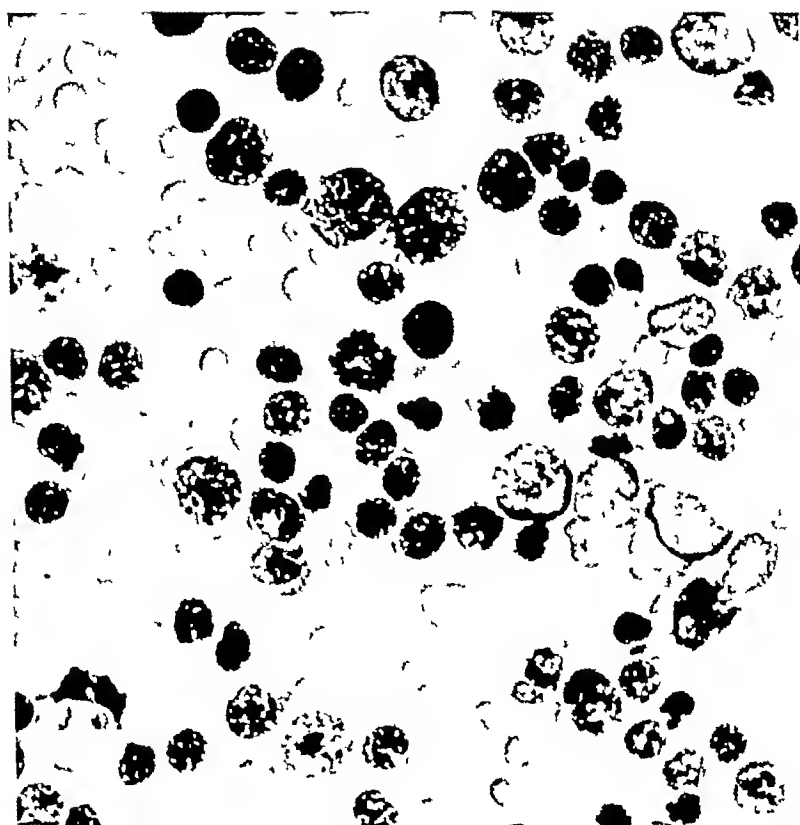


FIG 9 CASE 4 FOURTEENTH DAY OF CRISIS

Enormous increase of normoblasts Reticulocytes in peripheral blood 30 per cent

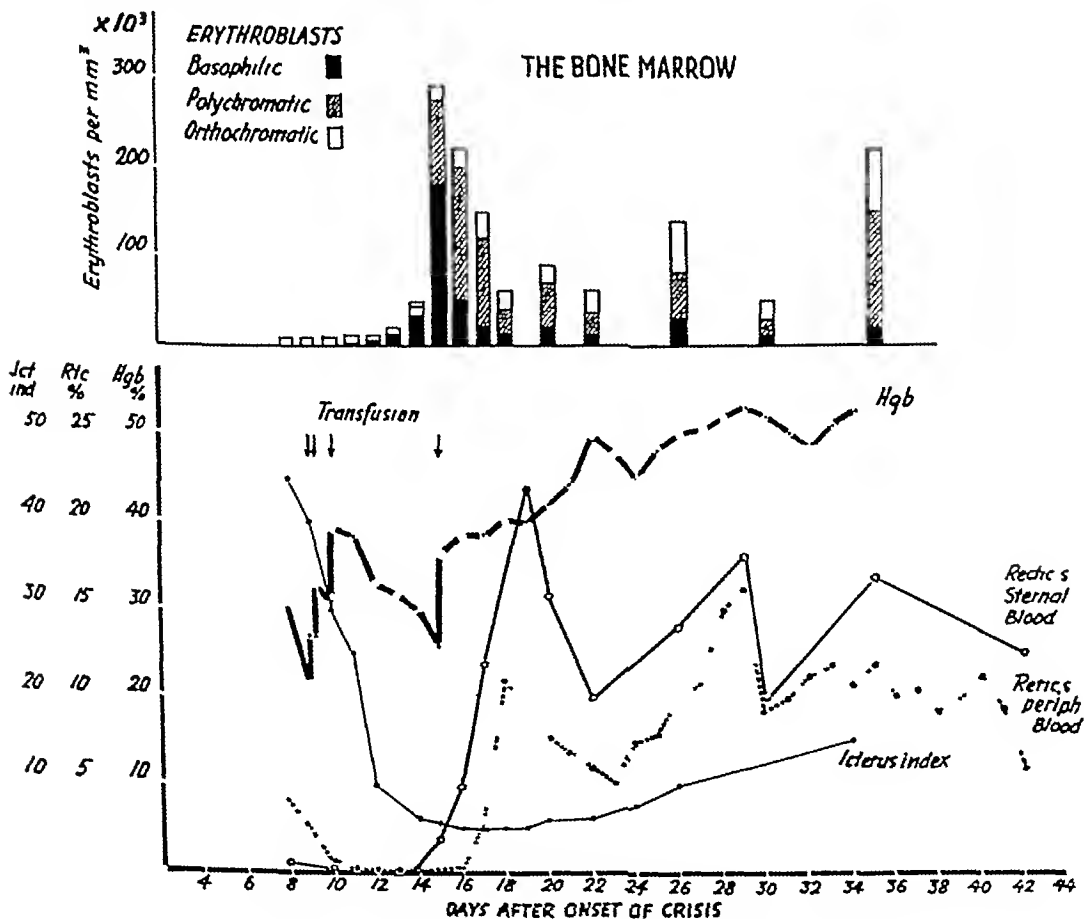


FIG 10 CASE 6 FEMALE 20 YEARS BLOOD AND MARROW FINDINGS



nucleated red cells is given in table 4, and the total number of nucleated red cells per cu mm of bone marrow is illustrated by the columns in figure 10. It follows that the crisis is accompanied by transient aplasia in the erythropoietic tissue as was demonstrated in the previous patient.

All 6 patients showed corresponding pictures. In table 5 are given the lowest blood values observed in the 6 patients during crisis. The reticulocytes disappear, severe anemia develops, granulocytopenia, and thrombocytopenia and the serum color is lowered to normal.

TABLE 4—Case 6 Number of nucleated cells and differential counts of nucleated red cells in bone marrow during crisis

	Days after onset of crisis														
	8	9	10	11	12	13	14	15	16	17	19	20	22	26	30
Erythropoiesis per cent	10.3	11.5	10.5	7.5	8.1	15.6	41.0	84.9	84.6	61.4	38.3	47.9	44.7	49.2	37.0
Leukopoiesis per cent	89.7	88.5	89.5	92.5	91.9	84.4	59.0	15.1	15.4	38.6	61.7	52.1	55.3	50.8	63.0
Nucleated cells in thousands per cu mm	107	102	90	115	90	120	115	345	256	220	160	188	136	284	134
Proerythroblasts	0.3	0.5	0.5	0.3	0.6	5.5	4.6	2.0	1.0	0.3	0.2	0.4	0.1	0.1	0.5
Macroblasts	1.5	1.0	1.6	2.1	2.5	5.0	22.8	51.5	17.5	5.6	3.0	8.0	7.0	11.0	5.8
Polychromatic normoblasts	—	—	—	—	—	0.5	9.8	27.4	56.4	38.9	21.0	30.1	18.1	16.0	16.0
Orthochromatic normoblasts	5.5	5.0	4.9	3.1	2.0	2.5	2.5	2.0	7.5	15.3	13.5	8.6	18.9	21.3	14.2
Pyknotic normoblasts	3.0	5.0	3.5	2.0	3.0	2.0	—	—	—	—	—	—	—	—	—
Mitotic normoblasts	—	—	—	—	—	0.1	1.3	2.0	2.2	1.3	0.6	0.8	0.6	0.8	0.5

TABLE 5—The lowest blood values observed during crisis

Case No.	Sex	Age	Hgb	RBC $\times 10^6$	Retics	WBC	Granulocytes	Thrombocytes	Icterus index
			%		%				
1	♀	8	30*	2.20	0.2	3,600	1,750	85,000	6
2	♂	59	20*	0.98	0.1	4,300	2,400	45,000	6
3	♂	40	28*	1.32	0.25	4,200	1,970	88,000	7
4	♂	38	36	1.57	0.0	2,800	1,200	30,000	5
5	♂	22	32	1.32	0.3	—	—	(106,000)	5
6	♀	20	21*	1.02	0.0	2,300	760	35,000	6

\* Patients treated with transfusions.

Figure 11 shows the reticulocyte counts in the 6 patients. On the whole the curves have the same form in that the reticulocytes disappear during the development of the anemia, and by recovery reticulocytosis appears in the peripheral blood ten to twelve days after the beginning of the crisis.

Another typical feature of the crisis is the diminution of the urobilin in the urine at the same time as the jaundice disappears, as seen in figure 12 from patient number 2.

Figure 13 from patient number 5, shows how the serum iron increases to pathologic value (in this case to 2007 per 100 ml) during the aplastic phase, subsequently to fall quickly. The concentration of uric acid in the blood also was in-

creased during the period of severe anemia up to 10 to 11 mg /100 ml (cf fig 13) and a fall to normal value with recovery

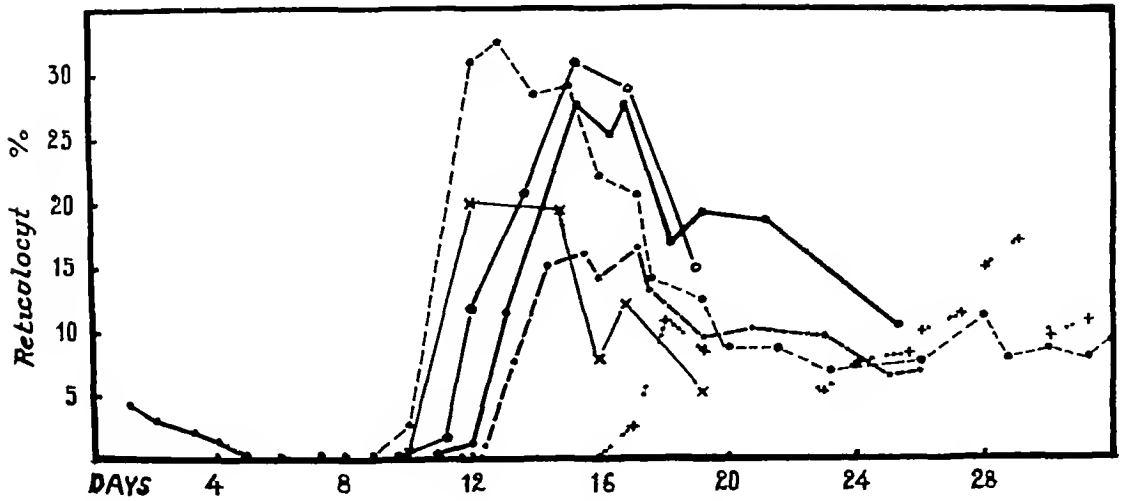


FIG 11 RETICULOCYTE COUNTS IN ALL 6 CASES

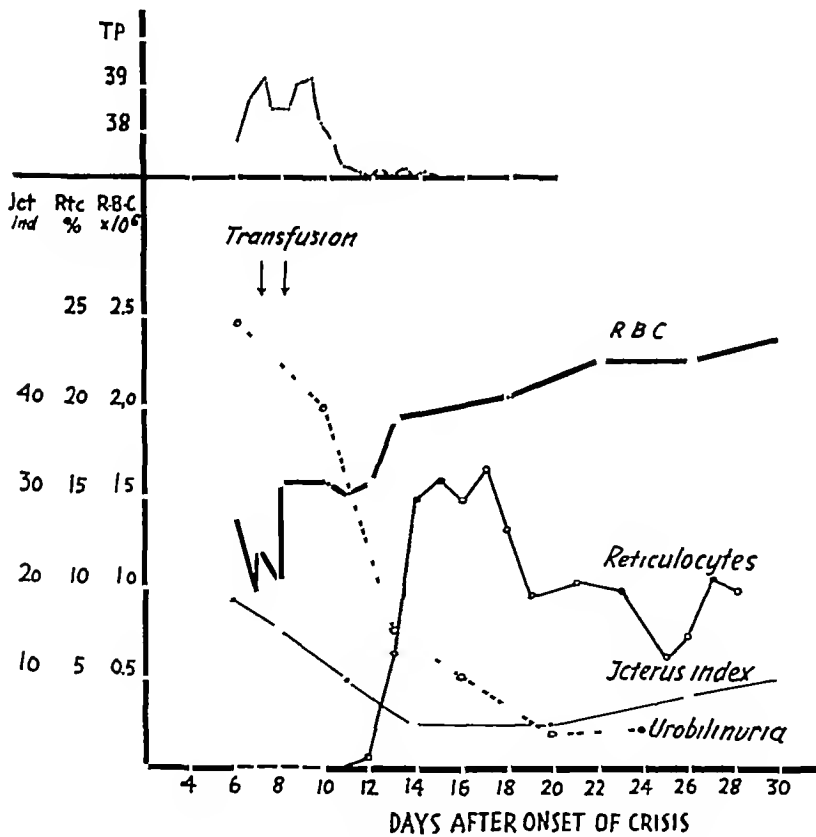


FIG 12 CASE 2 MALE 59 YEARS ILLUSTRATES THE DECREASING UROBILINURIA DURING THE CRISIS

If the patient is suffering from lack of iron during the period of rapid regeneration of hemoglobin, the iron deficiency may retard the regeneration. Figure 14 from patient number 6, shows a decrease of serum iron to 35 $\gamma$ /100 ml. The rise of reticu-

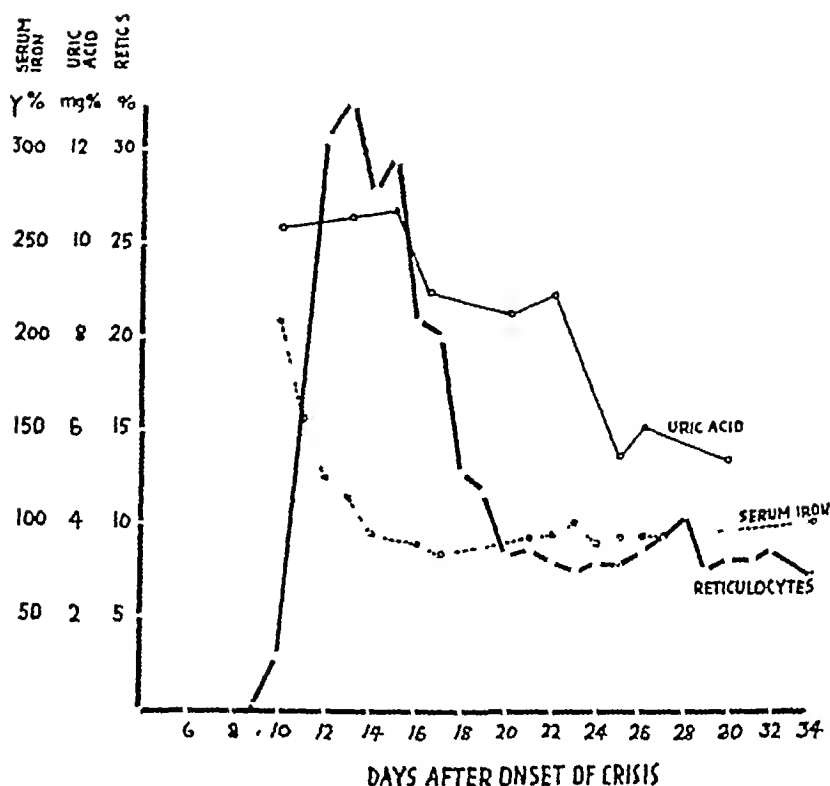


FIG 13 CASE 5 MALE 22 YEARS, THE VARIATION IN SERUM IRON AND URIC ACID DURING CRISIS

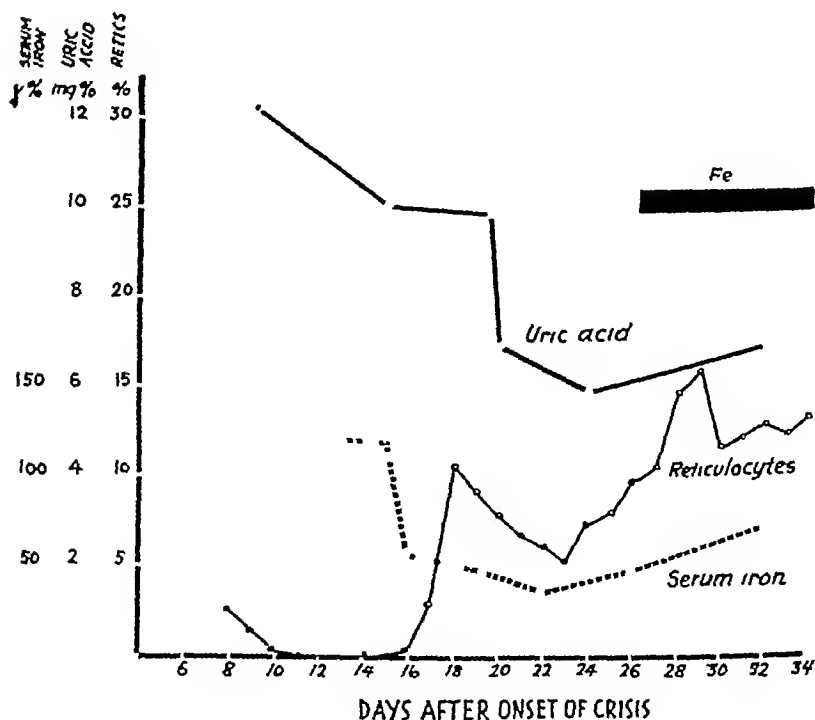


FIG 14 CASE 6 FEMALE 20 YEARS LOW SERUM IRON SECONDARY RISE OF RETICULOCYTES FOLLOWING IRON THERAPY

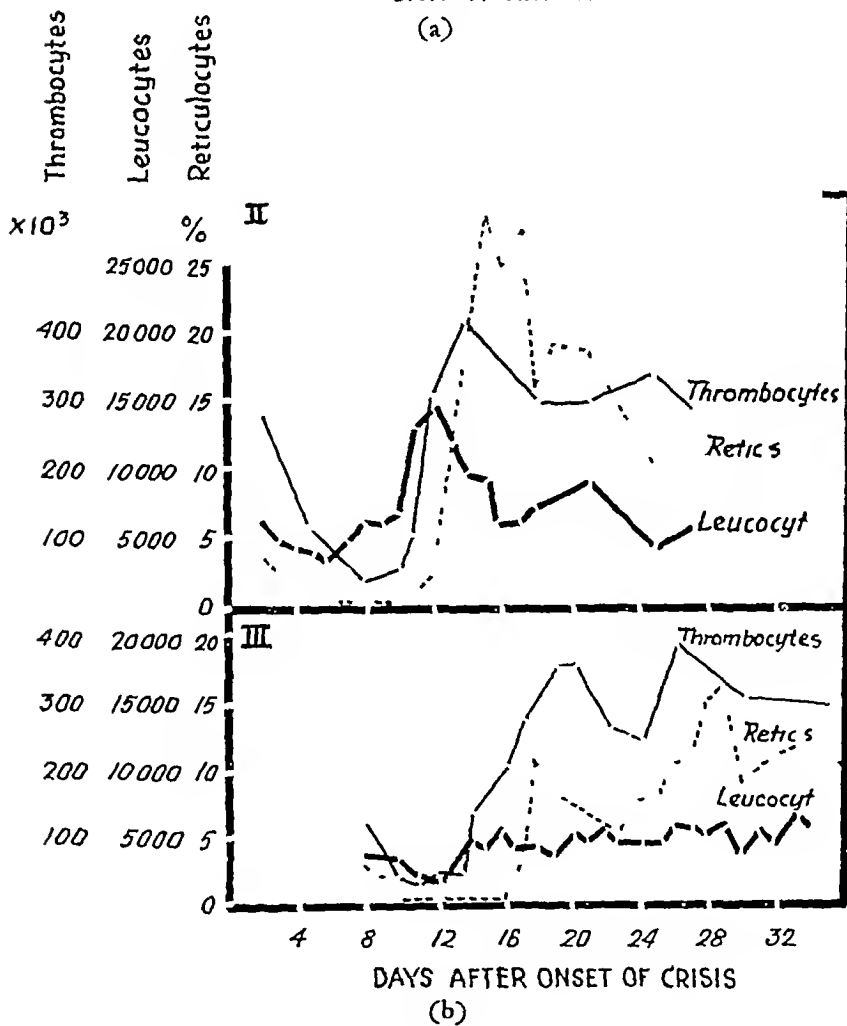
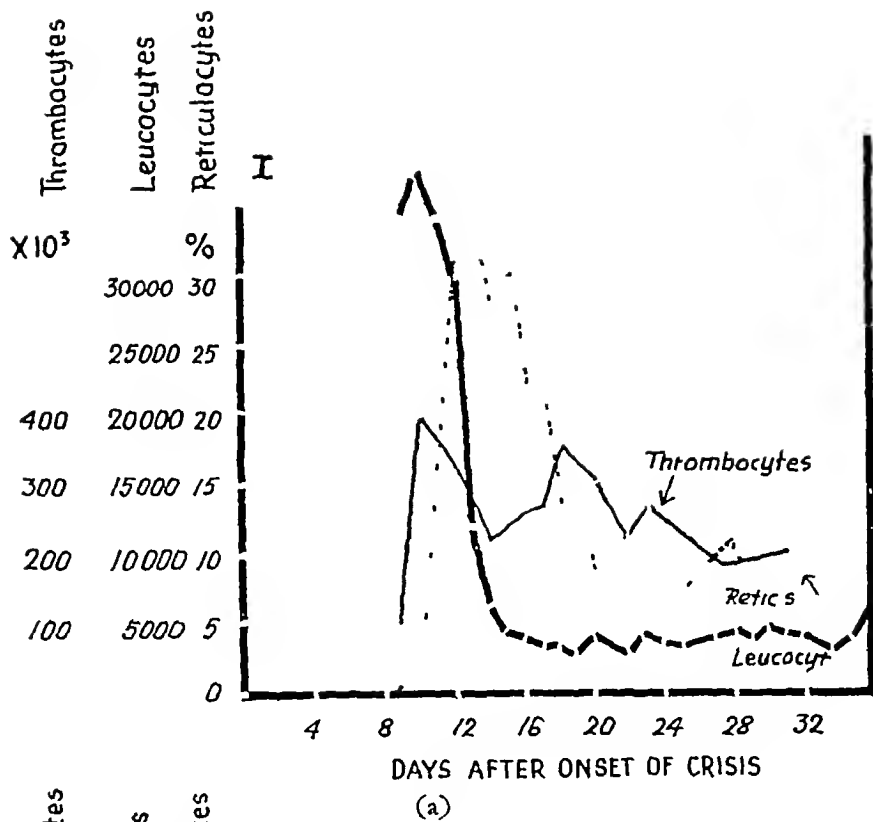


FIG 15 (a and b) THE VARIATION IN NUMBER OF LEUCOCYTES AND THROMBOCYTES DURING CRISIS

locytes in this case was lower and more prolonged. If iron is given at this stage, there is a secondary rise of reticulocytes and a more rapid improvement in the blood picture. The uric acid of the blood showed corresponding changes to those seen in the previous patient.

#### THE LEUKOPOIETIC AND THROMBOCYTOPOIETIC FUNCTION

As stated (cf. table 5) granulocytopenia and thrombocytopenia occur simultaneously with the development of anemia.

TABLE 6—Case 4 Differential counts of leukopoietic system

	8 days before crisis	Days after onset of crisis				
		4	6	9	12	14
Peripheral blood						
White cells	5,600	4,000	2,800	5,700	14,500	9,900
Granulocytes	3,920	2,200	1,200	3,990	12,100	8,200
Bone marrow leukoblasts and leukocytes						
per cent	47.2	95.2	95.2	71.0	19.0	40.0
number per cu. mm	127,000	85,700	67,100	89,000	72,000	87,000
Myelocytes	1.2	3.0	5.4	2.4	1.0	0.8
Promyelocytes	2.2	6.6	6.0	2.0	1.6	4.4
Neutrophilic						
Myelocytes	16.8	25.0	35.6	18.8	16.0	20.2
Metamyelocytes	30.6	15.2	13.2	18.0	15.2	14.8
'Band' forms	15.8	14.2	14.6	17.4	20.6	23.8
Polymorphonuclear	14.8	5.6	7.6	12.2	24.8	13.6
Eosinophilic granulocytes	3.2	2.2	2.4	2.8	2.0	1.8
Basophilic granulocytes	0.2	0.2	—	0.4	—	—
Lymphocytes	12.2	10.2	7.2	12.6	10.8	7.8
Monocytes	1.6	2.6	0.6	5.6	4.4	6.6
Plasma cells	0.8	0.2	4.6	0.4	0.2	0.2
Reticular cells	0.4	4.2	2.2	6.4	3.0	5.6
Megakaryocytes	0.2	0.8	0.6	1.0	0.4	0.4

The changes in the number of leucocytes and thrombocytes in the peripheral blood during the crisis are illustrated in figures 15a and b. In all cases the differential counts showed that the aplastic phase was accompanied by a granulocytopenia down to about 1000–2000 granulocytes per cu. mm (cf. table 5). The thrombocytopenia varied from 30 to 100,000 thrombocytes per cu. mm. In one of the patients (number 2) the thrombopenic phase was accompanied by a mild purpura. The granulocytopenic phase was not accompanied by any aplasia of the granulocytopoietic tissue. The number of leukoblasts and leukocytes per cu. mm of bone marrow was rather constant during the crisis as shown in table 2 and as may be calculated from table 4. The differential counts, however, showed a shift to the left during the granulocytopenic phase, with increasing number of promye-

lyocytes and myelocytes and decreasing number of mature forms. This event is illustrated in table 6 and points to the fact that the initial phase of the crisis apart from an erythropoietic aplasia is accompanied by an arrest of maturation of the leukopoietic tissue. The thrombocytopenia is likely to be explained in a similar way.

The number of lymphocytes and monocytes did not show any significant variation during the crises.

The recovery from crisis is heralded by leukocytosis as the first sign of improvement in the condition. The leukocytosis can attain a value up to 40,000 leukocytes per cu mm as in case 5 (cf. fig. 15a) and is accompanied by a shift to the left. A short time after the leukocytosis, an increase of thrombocytes appears, and finally the reticulocyte crisis occurs. This train of events was constant in all cases, and seems to bear a relation to the life cycle and rate of maturation of the respective elements of the blood.

A moderate azotemia was constantly found during the anemic phase. The concentration of blood urea increased to between 60 and 140 mg per 100 ml, and dropped to normal quite rapidly when the hemoglobin and red cell count began to increase.

#### DISCUSSION

The morphologic changes in the bone marrow which were demonstrated during crisis, together with the disappearance of the reticulocytes both from the peripheral and sternal blood, show that during the development of the anemia an acute aplastic condition is present in the erythropoietic tissue with complete cessation of the formation of red cells. The spontaneous recovery is characterized by a rapid regeneration accompanied by a reticulocyte crisis in the peripheral blood.

Can this transient aplasia in the erythropoietic tissue fully explain the development and symptoms of the crisis, or is there at the same time an increased hemolysis as it is usually assumed? As is stated before, there is no positive sign which points to increased hemolysis, on the contrary, the serum color and urobilinuria both decrease during the development of anemia. This was a constant finding in all cases, a condition which quite definitely contradicts the theory that an acute increase in the hemolytic processes is the reason for the crisis. The finding points to the fact that the total destruction has a tendency to decrease as the anemia increases. Thus, the formation of bilirubin is reduced, and the excretory capacity of the liver is sufficient to eliminate the coloring matter. The theory that jaundice increases during the crisis does not appear to be founded on objective investigations, and we believe it to be erroneous.

On the other hand, it is not a difficult matter to show that a sudden cessation in the formation of red cells in hemolytic jaundice will necessarily cause a rapid onset of anemia. When blood formation ceases, the rate at which anemia develops will be dependent on the lifetime of the red cells. Figure 16 shows that red cells from a patient with congenital hemolytic jaundice transfused to a normal individual, have a maximal lifetime of approximately fifteen days, while normal red cells transfused to a patient with hemolytic jaundice live about 110 - 120 days.

The test is carried out on another of our patients with compensated anemia, and the percentage of surviving red cells is determined with the help of Dacie and Mollison's (1943) modification of Ashby's (1919) differential agglutination technic. The result is in accordance with earlier investigations, among others, those carried out by Dacie and Mollison (1943). These transfusion experiments show that the fundamental pathologic disturbance in congenital hemolytic jaundice is associated with the actual red cells and not with any pathologically increased destructive function of the spleen or the remaining reticulo-endothelial system as maintained by Heilmeyer (1935), Bergenheim and Fåhræus (1936) and others. The determining feature of the short life span of the red cells is their spherocytic abnormality. There is reason to assume that this lifetime is the same in the patient as after transfusion to a normal individual.

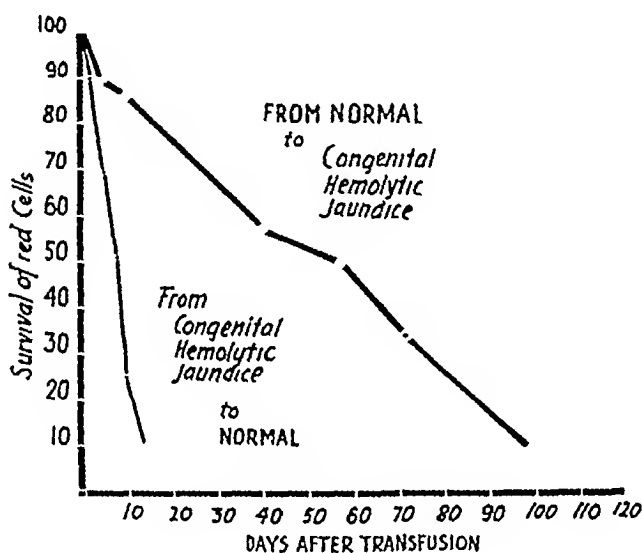


FIG 16 THE SURVIVAL OF ERYTHROCYTES FROM A CASE OF CONGENITAL HEMOLYTIC JAUNDICE, MAN AGED 38, TRANSFUSED TO A NORMAL RECIPIENT, AND THE SURVIVAL OF NORMAL ERYTHROCYTES TRANSFUSED TO THE PATIENT (FROM OWN INVESTIGATIONS TO BE PUBLISHED)

If we reckon an average lifetime of fifteen days in hemolytic jaundice, a sudden cessation in the formation will result in reduction in the number of circulating red cells to half in approximately seven to eight days, and in their total destruction in about fifteen days. On inspecting the curve of increasing anemia in figure 2, we find a similar course, with a reduction of the red cell count to about half in approximately six days, and if the curve is continued, total destruction in approximately fifteen days. This curve is an expression of the lifetime of the red cells in the patient as the formation is at a standstill, and we find that this completely corresponds to the lifetime of red cells from cases of hemolytic jaundice transfused to a normal individual (cf fig 16).

The development of the anemia, therefore, can be satisfactorily explained by the deficient blood formation, and there is nothing that points to the fact that these relapses are accompanied by any increase of the hemolytic or destructive processes. The remaining symptoms of the crisis also have a natural explanation from this point of view.

The increase in serum iron during the aplastic phase is a consequence of deficient consumption during this period, and the subsequent fall at the same time as the excessive hemoglobin formation sets in needs no further explanation.

The changes which are demonstrated in the granulocytes explain former incongruities on this point. Most hematologists have found leukocytosis as a typical feature in the symptomatology of the crisis, while others have found leukopenia. The varying findings are due to the investigations being carried out at different times during the development of the crisis. It ought to be emphasized further that the neutrophilic leukocytosis is only an expression of rapid regeneration in a similar manner, which can be seen after hemorrhages, and in liver therapy in pernicious anemia. This leukocytosis can not be made to account for any infectious etiology of the crisis as has been suggested.

The increase in the blood urea which occurs seems to be the result of a failing kidney function owing to the severe anemia, and the increase in uric acid in the blood may probably be related to the same disturbance.

Some earlier investigations have shown a very low reticulocyte count during crisis (Barber 1934, Murray-Lyon 1935), but these findings have not been further investigated. Lyngar (1942) found in one case, however, signs of reduced erythropoietic tissue in the bone marrow, and indicated a possibility of reduction in formation of the red cells as a link in the pathogenesis of the crisis. Dameshek (1941) has reported cases with leukopenia, thrombocytopenia and reticulocytopenia during crisis, and discusses these findings in the view of Heilmeyer's theory of a sudden splenic hyperactivity as the cause of the hemolytic crisis.

In conclusion, we can say from the investigations reported, that they support the opinion that congenital hemolytic jaundice is due to an abnormality in the red cells which makes them less resistant to the destructive function of the normal reticulo-endothelial system. The lifetime is short and the compensatory new formation is therefore active.

The crises are due to a sudden cessation in the formation of red cells and a depression of the formation of granulocytes and thrombocytes because of a transitory acute aplasia in the erythropoietic tissue together with a maturation arrest in the granulocytopoietic and thrombocytopoietic tissue. The crises ought, therefore, to be called aplastic crises, and not hemolytic crises.

The releasing factor of this acute aplasia is unknown, but the appearance of several cases together suggests that they can be produced by some extraneous reason, possibly infective.

#### SUMMARY

Six cases of congenital hemolytic jaundice with "hemolytic" crises are reported. It is demonstrated that during the development of the anemia an acute aplastic condition is present in the erythropoietic tissue of the bone marrow with complete cessation of the formation of red cells. The reticulocytes disappear from the blood, jaundice, serum bilirubin and urobilinuria decrease to normal values, and the serum iron increases. This period is further characterized by leukopenia and thrombocytopenia.



The spontaneous recovery is caused by a rapid regeneration of the erythropoietic tissue resulting in a marked reticulocytosis in the peripheral blood, and there is also leukocytosis, an increase in thrombocytes and a rapid fall of serum iron

During the period of severe anemia an increase in the blood urea and uric acid occurs

Transfusion experiments revealed an average lifetime of approximately fifteen days for the red cells in congenital hemolytic jaundice, a fact which fully explains the development and symptoms of the crisis as a result of cessation in the formation of red cells

The findings definitely contradict the theory that an acute increase in the hemolytic process is the reason for the crisis

The crisis should be called *aplastic* and not hemolytic

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## STUDIES ON THE PANCYTOPENIA OF KALA-AZAR

By CAPTAIN GEORGE E. CARTWRIGHT,\* M. C., A. U. S., HUI-LAN CHUNG, M. D.,  
AND AN CHANG, M. D.

IT HAS long been recognized that visceral leishmaniasis (kala-azar) is accompanied by marked changes in the peripheral blood, namely, pronounced leukopenia, anemia and thrombocytopenia<sup>1-3</sup>. Pathologically, the disease is characterized by a marked hyperplasia of the reticulo-endothelial system, particularly of the spleen, liver, bone marrow and lymph nodes. As a result of this reticulo-endothelial hyperplasia there is a marked enlargement of the spleen, and a moderate enlargement of the liver and lymph nodes. The bone marrow is hyperplastic and infiltrated with reticulo-endothelial cells, many of which are parasitized.

The anemia in kala-azar is moderately severe. Kuroya, Young, Tang and Hong<sup>4</sup> in a study of 151 cases in Kiangsu Province, China, found an average value of 2,930,000 red cells per cu. mm. The lowest count was 700,000. Keefer, Khaw and Yang<sup>5</sup> in a study of 191 cases in Peiping found the red count in the majority of patients to be between 2 and 4 million per cu. mm. Similar values have been obtained by Young in Peiping,<sup>6</sup> Botzaris in Greece,<sup>7</sup> Knowles,<sup>8</sup> Napier and Sharma,<sup>9</sup> Bramachari,<sup>3</sup> and Rogers<sup>10</sup> in India, and Mo in Manchuria.<sup>11</sup> In the majority of patients it has been reported that the hemoglobin is reduced in proportion to the erythrocyte count. Kuroya et al.<sup>4</sup> reported the mean color index in their 150 cases to be 0.9 with a range of 0.63 to 1.50. Similar results were obtained by Mo.<sup>11</sup> Napier and Sharma<sup>9</sup> have reported a mean of 0.885 in 47 Indian cases. Botzaris<sup>7</sup> found the color index to be low in 5 cases, normal in 2 and high in 2.

The size of the red cells in kala-azar has not been studied extensively. Napier and Sharma,<sup>9</sup> using an Eve halometer, report a mean diameter of  $7.559\ \mu$  in 22 cases prior to treatment and a mean diameter of  $7.375\ \mu$  following treatment. Kuroya, Young, Tang and Hong<sup>4</sup> studied the mean cell diameter in 35 cases. The values varied from  $7.03\ \mu$  to  $8.25\ \mu$  with an average of  $7.81\ \mu$  which they considered normal. In 28.6 per cent of the cases the mean diameters were increased, in 17.2 per cent reduced, and in 54.3 per cent normal. The Price-Jones curves showed a spreading of the base and a flattening of the peak. Curves having a tendency to shift to the right were comparatively numerous. The variation in width of the erythrocyte diameters was 5.2. Therefore, there was an abnormal amount of anisocytosis in most of the cases. There are no reports in the literature concerning mean corpuscular volume or mean corpuscular hemoglobin concentration.

Most authors agree that in kala-azar there is a slight reticulocytosis. In a series of 141 cases<sup>4</sup> values less than 10 per cent were recorded in 23 cases (16.3 per cent), 11-30 per cent in 39 cases (48.9 per cent), 31-50 per cent in 34 cases (24.1 per

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cent), and above 5.1 per cent in 15 cases (10.6 per cent). The mean value in this group was 2.8 per cent. In another series of 47 cases<sup>9</sup> the mean reticulocyte percentage was reported to be 2.07. Keefer, Khaw and Yang<sup>5</sup> state in reference to their 191 cases: "The reticulocytes are slightly increased, they are seldom above four per cent."

Normoblasts in the peripheral blood in kala-azar are not a constant or prominent feature. Napier and Sharma<sup>9</sup> report that in 47 cases with anemia no nucleated red blood cells were observed. Kuroya, Young, Tang and Hong<sup>4</sup> found normoblasts in the peripheral blood in only 26 of 151 cases examined. In 19 of these the normoblasts numbered 1 to 2 per 100 white cells, in 6 cases 3 to 6 per 100 white cells, while in one case the number reached 19. Keefer, Khaw and Yang<sup>5</sup> in a study of 191 cases state: "Signs of blood regeneration are usually lacking when one examines the peripheral blood. We have never seen nucleated red blood cells." Several authors have stated that normoblasts frequently appear in the blood but not in great numbers.<sup>6-8, 11</sup> It must be remembered that iron deficiency, a condition in which normoblasts appear in the peripheral blood, may have been a complicating factor in some of the patients.

Polychromatophilia, stippling and poikilocytosis are rarely observed in kala-azar.<sup>4, 7</sup> Polychromatophilia has been reported in 28 out of 151 cases but then only in small numbers.<sup>4</sup> Stippling was seen in only one case. Knowles<sup>8</sup> and Botzaris<sup>7</sup> reported a moderate degree of poikilocytosis but Kuroya et al.<sup>4</sup> were unable to confirm this.

The characteristic leukopenia in kala-azar was first described by Rogers in 1904.<sup>10</sup> He stated that if the proportion of leukocytes to red corpuscles is less than 1:1500, the patient is probably suffering from kala-azar. Manson-Bahr<sup>12</sup> states that the reduction in the number of leukocytes is persistent and that usually the proportion of leukocytes to red blood corpuscles is reduced to 1:2000-4000. The leukopenia has subsequently been confirmed by many authors.<sup>4-8, 11</sup> Kuroya, Young, Tang and Hong<sup>4</sup> in their cases found the total leukocyte count below 1000 in 2.7 per cent, 1000-2000 in 21.9 per cent, 2000-3000 in 34.4 per cent, 3000-4000 in 19.2 per cent, 4000-5000 in 13.2 per cent and in only 8.6 per cent was the leukocyte count above 5000. The lowest count was 740 and the highest 11,740. The average leukocyte count was 3052 per cu. mm. This is slightly lower than that reported by Young,<sup>6</sup> Knowles<sup>8</sup> and Mo.<sup>11</sup>

The leukopenia of kala-azar is due mainly to a neutropenia. Both the percentage and the absolute number of neutrophilic leukocytes are markedly reduced. There is a distinct shift to the left as far as the number of juvenile neutrophils is concerned but in one series of 151 cases myelocytes were seen in only one case.<sup>4</sup> Toxic granules are very few. The eosinophils generally decrease in numbers or disappear entirely from the blood. Basophils occur in normal numbers. Plasma cells are occasionally observed. The percentage of lymphocytes is generally increased to about 50. The absolute number of lymphocytes is moderately reduced. Several authors have reported that both the percentage and the absolute number of monocytes show an increase.<sup>6-8</sup> On the other hand, other authors state that the monocytes show an increased percentage, but the absolute count is normal or less than normal.<sup>10, 12</sup>

Kuroya, Young, Tang and Hong<sup>4</sup> found that the monocytes were less than 10 per cent in 71 per cent of their cases. In only 8.6 per cent was the monocyte count above 15 per cent. Similar findings have been reported by Botzaris.<sup>7</sup>

From the differential white cell studies reported it can be concluded that the characteristic leukopenia of kala-azar is mainly due to the great reduction of neutrophilic leukocytes, the other classes of leukocytes playing only a minor role. Acute agranulocytosis has been reported to occur in patients with kala-azar.<sup>13, 14</sup>

One of the outstanding clinical features of kala-azar is a hemorrhagic tendency. Epistaxis and bleeding from the mucous membranes are frequent although frank purpura is rare. The hemorrhagic tendency resembles purpura hemorrhagica in that there is a prolonged bleeding time, a normal coagulation time, a nonretractile clot, and in some instances a positive tourniquet test. Darling<sup>15</sup> in 1911 was the first to comment on the change in blood platelets when he stated: "Judging from films from the peripheral blood and spleen, there is a very marked reduction in the number of platelets." In 1930 Yang and Ch'en<sup>16</sup> confirmed Darling's observation. The platelets in their 23 cases varied from 50,000 to 100,000 per cu. mm. Kuroya, Young, Tang and Hong<sup>4</sup> performed platelet counts on 117 patients. The count was below 40,000 per cu. mm. in 23 per cent, between 40,000 and 80,000 in 42 per cent, between 80,000 and 120,000 in 18 per cent, between 120,000 and 160,000 in 11 per cent, and above 160,000 in 6 per cent with an average for all cases studied of 76,763 per cu. mm.

Detailed studies of the bone marrow in kala-azar with differential cell counts have not been reported. Most of the studies are only gross estimates obtained from postmortem specimens stained with hematoxylin-eosin. These studies for the most part have been made on patients in the advanced stage of the disease. Differential cell studies done on thin smears stained with Wright's stain and taken in the early stages of the disease are lacking. Consequently, there is considerable discrepancy in the literature as to the changes in the marrow. Chatterjee,<sup>17</sup> on the basis of the postmortem femoral bone marrow studies of hematoxylin-eosin preparations in 6 patients, divides the marrow into three stages. In the first and earliest stage a differential count gave the following proportion of cells: myeloid, 23 per cent, erythroid, 25 per cent, clasmatoocytes, 45 per cent, other cells, 7 per cent. About 30 per cent of the clasmatoocytes were parasitized. In the second stage the total number of cells had become fewer and the differential count of one case was as follows: myeloid, 30 per cent, erythroid, 40 per cent, clasmatoocytes, 5 per cent, other cells, 25 per cent. Almost all of the clasmatoocytes were parasitized. In the third, or more advanced stage, the total number of cells had become even fewer, the cells of the myeloid series being diminished. A differential count from one case was as follows: myeloid cells, 11 per cent, erythroid, 50 per cent, clasmatoocytes, 10 per cent, other cells, 29 per cent. Many of the erythropoietic cells, it is stated, were megaloblasts. This finding led the author to state: "The appearance of megaloblasts and a large number of erythroblasts may be associated with the increasing anemia which occurs in this condition and which might ultimately turn into an anemia of the macrocytic type." Piney,<sup>18</sup> on the other hand, states that the erythroid cells are

markedly decreased to less than 8 per cent and that there is a marked increase of the lymphocytes and monocytes in the marrow to about 55 per cent. Meleney<sup>19</sup> studied the rib marrow in one case of kala-azar in man and reported "This marrow contains no fat, and approximately 80 per cent of the space is occupied by clasmatocytes, packed closely together into definite clasmatocyte tissue. The normal hematopoiesis is almost entirely absent, especially erythropoiesis. Most of the cells aside from the clasmatocytes seem to be lymphocytes. There are a few myelocytes and megakaryocytes." Hu,<sup>20</sup> in a somewhat more extensive, although still not a detailed study of the bone marrow in human kala-azar, reported similar changes. The marrows were hyperplastic and contained many reticulo-endothelial cells. The number of myelocytes was increased, but the metamyelocytes and leukocytes were either greatly reduced in number or entirely absent. The number of normoblasts was moderately increased in a few cases, but in most instances the number was either normal or decreased. Plasma cells were prominent. The megakaryocytes were reduced from 3 to 4 per high power field to an average of about 2 per high power field. Hu and Cash<sup>21</sup> studied the bone marrow of two patients coming to autopsy and report "The bone marrow of the femur consisted in a solid mass of firm, dark, grayish red tissue, which microscopically was found to consist mainly of all forms of myeloid cells and nucleated red blood corpuscles. Such a degree of hematopoietic activity is rarely seen other than in cases of pernicious anemia. Many megakaryocytes and a number of large phagocytic cells similar to those seen in the spleen were present."

In hamsters<sup>22-23</sup> and squirrels<sup>24</sup> experimentally infected with kala-azar the bone marrow is hyperplastic due to an increase in the number of reticulo-endothelial cells. Chung and Ch'in<sup>24</sup> state "In squirrels infected with kala-azar the bone marrow shows the most marked hyperplasia of the reticulo-endothelial cells, as compared with other organs. About three-fourths or more of the marrow space is occupied by these cells. It is true that all of the blood elements of an active marrow are present. But when one considers the preponderance of the reticulo-endothelial cells, one cannot get away from the impression that the myelogenic and erythrogenic elements are actually being crowded out by the reticulo-endothelial cells. In fact, these cells show such an active proliferation that they come to resemble a malignant tumor growth invading or crowding out the marrow tissue." Meleney<sup>19</sup> has reported similar findings in hamsters.

The pathogenesis of the pancytopenia is poorly understood. Most students of the disease have offered the theory,<sup>4-5-11-19-20-22-24</sup> and it has been generally accepted, that the hematologic changes are the result of impairment of the hematopoietic function of the bone marrow which is destroyed mechanically by the overgrowth of parasitized reticulo-endothelial cells, that is, myelophthisic anemia. The evidence for this rests solely in the demonstration of numerous reticulo-endothelial cells in the marrow and much of this evidence has been obtained from the animal experiments mentioned above. Napier and Sharma<sup>9</sup> suggested that the anemia is due to excessive hemolysis. They based their conclusions on the slight reticulocytosis, the presence of an indirect blood Van den Bergh reaction, and the presence of urobilinuria. It has since been suggested that the urobilinuria

is due to hepatic insufficiency <sup>20</sup> Mo<sup>11</sup> has pointed out that the erythrocyte fragility is normal. Furthermore, the hemolytic theory does not explain the leukopenia and thrombocytopenia.

It is now recognized that in many conditions in which there is enlargement of the spleen due to hyperplasia of the reticulo-endothelial system there is an accompanying anemia, leukopenia, thrombocytopenia and hyperplasia of the bone marrow <sup>25-27</sup> Following splenectomy the cellular elements of the blood and bone marrow rapidly return to normal. These manifestations have been grouped together under the term "hypersplenism." Any one (thrombocytopenic purpura, congenital hemolytic icterus, primary splenic neutropenia) or all (primary splenic pancytopenia) of the cellular elements of the blood may be affected. The pathogenesis of the hematologic changes is in dispute. One group<sup>26</sup> holds that a hormonal relation exists between the spleen and the bone marrow. The other<sup>25</sup> fosters the "phagocytic hypothesis," in which the anemia, leukopenia and thrombocytopenia are thought to be due to increased phagocytic activities of the enlarged spleen. Since in kala-azar there is marked proliferation of the reticulo-endothelial system and consequent splenomegaly, the possibility that the blood changes are due entirely or at least in part, to hyperfunction of the spleen must be seriously considered. Splenectomy has been reported to be beneficial in kala-azar<sup>28, 29</sup> but unfortunately the blood changes were not followed closely and the patients received antimony prior to splenectomy. A careful study of the effect of this procedure alone on the blood has not been reported.

### MATERIALS

The case material for this study consisted of 143 patients with kala-azar. Twenty-five of these have been previously reported by one of us (H. L. C.)<sup>36</sup> All patients gave a history characteristic of the disease. None had received specific therapy prior to study. The diagnosis in each case was proved by the demonstration of *Leishmania donovani* in material from either sternal marrow puncture or splenic puncture. One-hundred and twenty-four cases were uncomplicated.

Twenty-seven patients were studied in detail and this group comprises the main body of this report. All of these except 5 were hospitalized for study. The significant clinical data for this group are presented in table 1. Eighteen of the patients had uncomplicated kala-azar. In general, the majority of these patients were in the intermediate stage of the disease. Follow-up studies after the onset of specific therapy were available in 12 patients.

### METHODS

*Blood Counts* These were performed in the usual manner according to Wintrobe.<sup>30</sup>

*Hemoglobin* For these determinations the Sahli hemoglobinometer was used.

*Peripheral Blood Smears* Smears were prepared by the cover-glass technic<sup>30</sup> and stained with Wright's stain. Differential white cell counts were performed on 100 cells, and the morphology of the red cells noted.

*Peripheral Platelet Counts* The counts were made indirectly by determining the

number of platelets per 1000 red cells in the blood smear. The number of platelets per cu mm of blood was then calculated from the red count which was done at the same time. In normal Chinese we found the platelet count to vary between 288,000 and 384,000 with an average of 330,000 per cu mm.

**Globulin Test** From an ordinary skin puncture 20 cu mm of blood was taken into a hemoglobin pipet and transferred immediately into a clean test tube (7-8

TABLE I — *Clinical Data*

Patient	Age in Years	Sex	Duration of Symptoms in Months	Size of Liver cm *	Size of Spleen cm *	History of Bleeding Tendency	Globulin Test	Comment
1	9	F	2	5	9	—	4+	Uncomplicated
2	2	M	2	3	4	—	4+	Uncomplicated
3	4	F	4	2	6	+	3+	Uncomplicated
5	6	M	7	7	12	+	4+	Uncomplicated
6	26	F	1	2	6	+	3+	Uncomplicated
7	11	M	7	5	13	+	4+	Uncomplicated
8	2	M	4	3	6	+	3+	Uncomplicated
9	15	F	1	3	4	—	3+	Uncomplicated
10	18	M	4	4	18	+	2+	Uncomplicated
11	12	F	1	0	1	—	1+	Uncomplicated
15	28	M	6	5	11	—	4+	Uncomplicated
19	34	F	4	5	7	+	4+	Uncomplicated
22	9	F	1	4	8	—	3+	Uncomplicated
23	13	M	1	3	5	—	0	Uncomplicated
24	20	M	8	6	18	+	4+	Uncomplicated
25	8	M	24	1	10	+	4+	Uncomplicated
26	23	M	3	2	8	+	3+	Uncomplicated
27	57	F	1	5	5	—	3+	Uncomplicated
4	10	F	3	2	7	+	3+	Noma, mild
12	28	M	?	9	15	—	4+	Tuberculosis, active, Noma, moderate
13	10	M	12	0	13	+	3+	Noma, moderate
14	2	F	5	3	7	+	3+	Noma, severe
16	2	M	2	3	4	+	0	Bronchitis, severe
17	3	M	6	4	8	+	0	Pneumonia, died
18	5	M	5	3	9	+	4+	Noma, severe
20	7	M	10	2	15	+	4+	Noma, severe
21	2	F	3	5	2	—	4+	Pneumonia, died

\* Measured in cm below costal margin in the mid-clavicular line

F—Female, M—Male

mm in diameter) containing 0.6 cc of distilled water. The contents of the tube were mixed by whirling in the hand. The tube was then allowed to stand vertically without further shaking and examined at intervals of 5, 15, 30 and 60 minutes. A haziness at the end of 5 minutes was taken to indicate a positive test. A definite settling of the precipitate within 15 minutes was recorded as 4 plus, definite settling of the precipitate within 15 to 30 minutes as 3 plus, definite settling between 30 and 60 minutes as 2 plus, and a haziness with a fine precipitate but no sediment after one hour as one plus.

**Spinal Puncture** This was performed by the introduction of a large lumbar-puncture needle into the marrow space. A small amount of marrow fluid, usually less than 0.3 cc., was withdrawn into a clean dry syringe and thin cover-glass

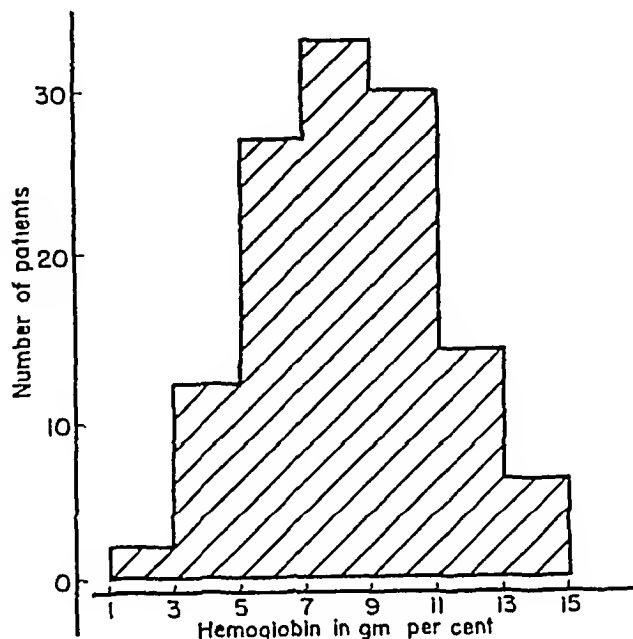


FIG. 1. Showing the degree of anemia in 124 patients with uncomplicated kala-azar

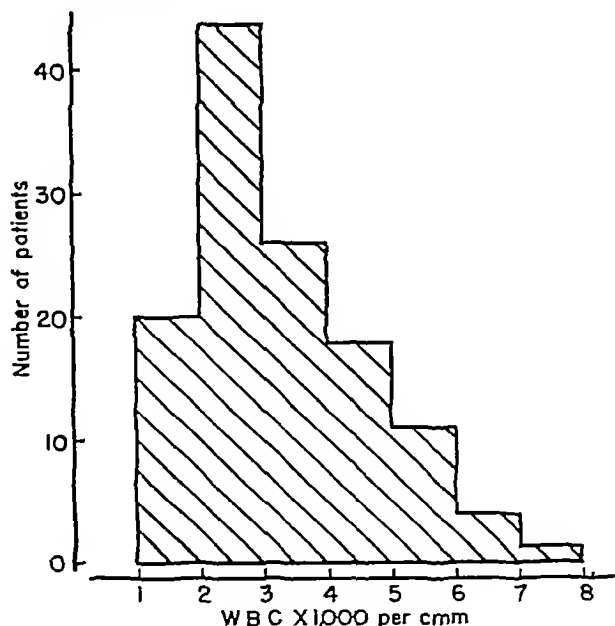


FIG. 2. Showing the leukocyte count in 124 patients with uncomplicated kala-azar

preparations drawn and stained with Wright's stain. The cover-glasses were then fixed to glass slides with neutral Canada balsam. After examining three such preparations the percentage of reticulo-endothelial cells was estimated. Because of the tendency of these cells to clump in the thick sections of the preparations and because of their extreme fragility in the thin sections precise counts were not possible. The percentage of these cells parasitized was also estimated. Differential



counts on the bone marrow cells were done on 500 to 1000 cells, exclusive of reticulo-endothelial cells and megakaryocytes, and the percentage of each accurately determined. The total number of megakaryocytes per million nucleated cells was determined by pasting a 10 x 10 mm square cut out of paper over each of three cover-glass preparations. This area represented approximately 8000 oil immersion fields. The number of nucleated cells in each area was estimated by accurately enumerating the nucleated cells, including the reticulo-endothelial

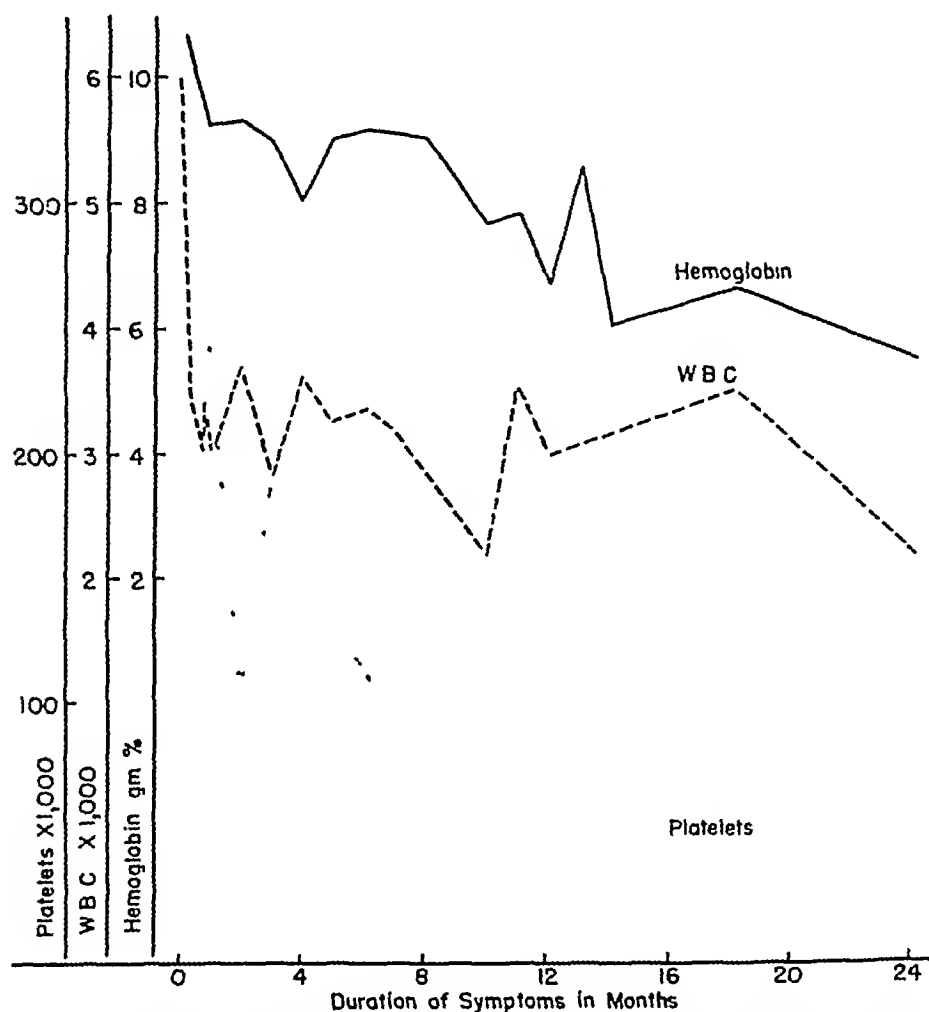


FIG 3 Showing the relation between the duration of symptoms and the hemoglobin (116 patients) leukocyte count (108 patients) and platelet count (36 patients) in uncomplicated kala-azar

cells, in three oil immersion fields in each corner and the center. The megakaryocytes in each of the three 10 x 10 mm squares were counted and the number per million nucleated cells calculated and averaged. To determine the percentage of megakaryocytes forming platelets at least 50 megakaryocytes were counted.

The values for the relative numbers of nucleated cells in the normal bone marrow were taken from Wintrobe.<sup>30</sup> These values agree closely with those found by us in normal marrow. The total number of megakaryocytes per million nucleated cells was determined in 10 normal marrows. The values were found to range from 85 to 200 with an average of 142. The percentage of megakaryocytes forming platelets in normal marrow was found to vary from 55 to 87. The average percentage was 75.

In attempting to interpret the correlation between the duration of the symptoms and the blood counts (fig 3), the size of the spleen and the blood counts (fig 4)

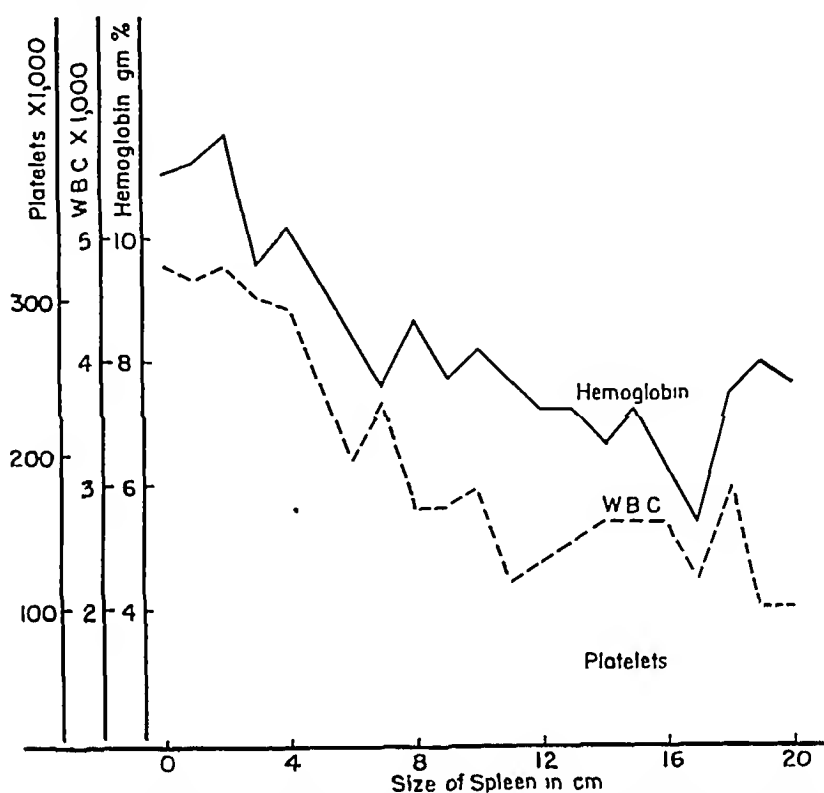


FIG 4 Showing the relation between the size of the spleen and the hemoglobin (117 patients), leukocyte count (117 patients) and platelet count (36 patients) in uncomplicated kala-azar

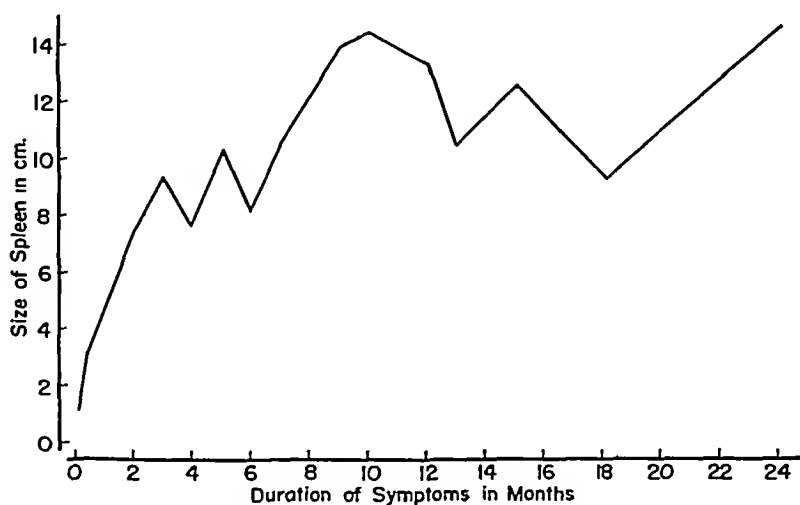


FIG 5 Showing the relation of the size of the spleen and the duration of symptoms

and the duration of symptoms and the size of the spleen (fig 5) certain considerations must be kept in mind. Many patients were unable to remember clearly the exact time of onset of symptoms. The size of the spleen was measured in one diameter only and the size of the spleen in relation to the size of the patient was not considered. Characteristic of kala-azar are remissions and relapses rather than

a gradually progressive course. The curves are plotted from a large body of data. They are shown to represent the general trend. A particular patient might not be expected to fit exactly in this scheme.

## OBSERVATIONS

### A PERIPHERAL BLOOD

1 *Anemia* The distribution of the hemoglobin values in 124 uncomplicated cases is shown in figure 1. The average value for the entire group was 8.3 grams per cent. The anemia at times was severe. In 33 per cent of the patients the hemoglobin was less than 7 grams per cent. As shown in figure 3, significant anemia (10.6 Gm. per cent) was usually present shortly after the onset of symptoms and was thereafter gradually progressive, reaching an average value of about 5 grams per cent in 24 months if the disease remained untreated. In patients with kala-azar and an associated bacterial infection the anemia was more severe (average for 19 patients, 7.2 grams per cent). The correlation between the size of the spleen and the degree of anemia is demonstrated in figure 4. By the time the spleen had become palpable there was a significant degree of anemia (11.2 Gm. per cent hemoglobin) and thereafter the anemia increased in proportion to the splenic enlargement.

2 *Red Cell Morphology* The red cell morphology was studied in 27 cases (table 2). Facilities were not available for determining the mean corpuscular volume. Judging from the appearance of the red cells in the thin smears the anemia was normocytic. The average color index for the group was 1.0. A significant degree of macrocytosis was observed in only two smears and complications were present in both patients. Neither microcytosis nor hypochromia were observed. A moderate amount of anisocytosis was generally present but poikilocytosis of significant degree was observed in only 4 of the uncomplicated cases. Polychromatophilia and stippling were not seen in uncomplicated kala-azar whereas a significant degree of polychromatophilia was seen in 3 of the 9 patients with complications. Nucleated red cells were observed in the peripheral blood in only 5 of the 27 patients and then never in large numbers. Three of the 5 patients with nucleated red cells in the peripheral blood presented complications.

3 *Leukopenia* As demonstrated in table 2 and figure 2, leukopenia was an almost constant feature of uncomplicated kala-azar. The average white cell count for the group of 124 patients was 2835 per cu. mm. The highest white count observed was 7250. Only 16 of the patients (12.9 per cent) had a white count above 5000 per cu. mm., all of these being early cases. Leukopenia was invariably present in late uncomplicated kala-azar. The leukopenia developed early, about the time of the onset of symptoms, and thereafter became only slightly more severe (fig. 3). The reduction in the white count appears to correspond closely to the degree of splenic enlargement (fig. 4). Leukopenia was usually present at the time the spleen was first palpable.

The white count in the patients with bacterial infections in addition to kala-azar was variable (table 2). Leukopenia was present in 4, a normal leukocyte count in 3 and a leukocytosis in 2. The latter response is demonstrated in figure 6.

Following treatment of the severe noma with penicillin the white count fell from 18,000 to 4000 per cu mm and after specific antileishmanial therapy returned to 6200

 TABLE 2 — *Peripheral Blood Studies*

Patient	RBC Million/ c mm	Hemo- globin Gm / 100 cc	W B C per c mm	Platelets thou sands/c mm	Differential W B C							R B C Morphology*					
					Myelocytes	Metamye- locytes	Neu- tro- phils	Eosinophils	Basophils	Lym- pho- cytes	Monocytes	Nucleated R.B.C	Size	Anisocy- tosis	Poikilo- cytosis	Polychroma- tophilia	Stippling
1	2 12	6 8	2450	105	0	7	16	0	0	67	10	0	N	1+	0	0	0
2	4 45	13 0	4100	98	0	3	15	1	0	75	6	0	N	2+	1+	0	0
3	5 85	13 9	4500	128	0	4	18	1	0	66	11	0	N	1+	0	0	0
5	2 88	11 8	4000	105	1	5	29	2	1	47	15	0	N	1+	0	0	0
6	3 10	9 4	2750	175	0	5	52	0	0	35	8	0	N	0	0	0	0
7	3 10	9 0	2350	65	0	2	30	0	0	55	13	0	N	0	0	0	0
8	2 10	6 0	2950	109	0	2	19	0	0	66	10	3	N	1+	0	0	0
9	3 74	9 4	4100	235	0	2	37	0	0	58	3	0	N	0	0	0	0
10	3 25	9 2	3100	110	0	1	70	0	0	23	6	0	N	1+	1+	0	0
11	4 45	12 5	3500	267	0	5	48	0	1	43	3	0	N	1+	0	0	0
15	3 68	10 2	1900	122	0	8	20	0	0	69	3	0	N	1+	0	0	0
19	1 56	5 8	1700	104	0	4	36	0	0	46	14	0	N	1+	0	0	0
22	2 05	7 4	2000	256	0	6	12	0	0	71	10	1	N	3+	1+	0	0
23	3 12	9 4	2300	250	0	14	40	0	0	32	14	0	N	1+	0	0	0
24	3 40	10 0	1500	96	0	4	45	0	0	36	15	0	N	1+	0	0	0
25	2 23	6 2	3050	127	0	5	18	0	0	68	9	0	N	2+	1+	0	0
26	2 80	9 9	1600	180	0	10	36	0	0	49	5	0	N	1+	0	0	0
27	4 12	11 0	2300	247	0	15	48	0	1	33	3	0	N	1+	0	0	0
Aver	3 22	9 5	2786	154	0	5 7	32 7	0 2	0 2	52 2	8 8	0 2	N	1+	0	0	0

## Patients with Complications

4	3 56	11 0	2500	187	0	5	38	0	0	47	10	0	N	1+	0	0	0
12	2 54	7 2	2800	189	0	9	51	0	0	30	10	0	N	0	0	0	0
13	1 36	4 7	3700	34	0	6	43	0	0	42	7	2	N	2+	0	1+	0
14	2 01	5 0	6500	140	0	3	22	0	0	29	45	1	M	2+	2+	2+	0
16	2 12	9 0	8600	113	1	7	30	0	1	57	3	1	M	2+	0	2+	0
17	3 12	10 0	12100	109	0	14	53	0	0	25	8	0	N	1+	1+	0	0
18	3 06	9 6	18800	144	0	5	59	0	0	31	5	0	N	0	0	0	0
20	2 46	7 7	6300	158	0	4	42	0	0	45	9	0	N	3+	1+	0	0
21	0 81	3 0	3400	51	0	6	33	0	0	55	6	0	N	1+	0	0	0

\* N—Normocytic, M—Macrocytic

4 *Differential Leukocyte Count* The most conspicuous change in the differential count was a reduction in the neutrophils (table 2.) In uncomplicated cases the neutrophils were reduced to an average of 32.7 per cent. This was a reduction in the absolute number from about 4000 to 910 per cu mm. A significant "shift" of the nuclei, either to the left or to the right, was not noted. The proportion of

metamyelocytes was not increased significantly and the absolute number was reduced by less than one-half. A single myelocyte was seen in two smears. Toxic granules in the neutrophils were not evident. Plasma cells were occasionally seen. Although the percentage of lymphocytes was increased to an average of 52.1 per cent, the absolute number of lymphocytes was reduced from the normal of about 2100 to 1450 per cu mm. The percentage of monocytes was slightly above normal but again the absolute number was reduced (2.45 per cu mm average). Eosinophils were seen in only 3 smears and then not above 2 per cent. Basophils were also rarely seen.

5. *Platelets* The platelet count in the majority of the patients was reduced (table 2). In the uncomplicated cases the average platelet count was 103,000 per cu mm, as compared with the normal average value of 330,000 per cu mm obtained by us under the conditions of this study. As seen in figure 3 the thrombocytopenia developed somewhat later than the anemia and leukopenia. Significant thrombocytopenia was generally not present until about two months following the onset of symptoms. After this time the platelet count fell rapidly. As the spleen enlarged the platelet count became progressively lower (fig. 4). A prolongation of the bleeding time and in most cases a positive tourniquet test were associated with the thrombocytopenia, although frank purpura was present in only one of the 143 patients. When complicating infections were present the thrombocytopenia was generally more severe. In one patient (no. 21), critically ill with lobar pneumonia, the platelet count was reduced to 51,000 per cu mm and the bleeding time was increased to 30 minutes. Another patient (no. 13), with severe noma, had a platelet count of only 34,000 and a bleeding time of seven minutes. However, in one patient with complicating diphtheria the platelet count was 306,000 per cu mm even though symptoms of kala-azar had been present for six months.

## B BONE MARROW

1. *Hyperplasia* The bone marrow in kala-azar was consistently hyperplastic. Thin smears and imprints of sternal marrow were exceedingly cellular. Globules of fat were rarely seen. Indeed it was even difficult to obtain a thin smear because of the cellularity of the marrow. Biopsy specimens of the sternal marrow stained with hematoxylin-eosin contained little fat and showed hyperplasia of the reticulo-endothelial tissue as well as blood forming tissue.

2. *Reticulo-endothelial Cells* Much of the hyperplasia in the sternal marrow could be accounted for by the increase in reticulo-endothelial cells. These cells, it was estimated, constituted from 5 to 50 per cent of all the cells in the sternal marrow (table 3). In about half the cases less than 1 per cent of the reticulo-endothelial cells were parasitized but in one patient (no. 20) as many as 60 per cent were estimated to be parasitized. Even in the marrows with 40 to 50 per cent reticulo-endothelial cells, large numbers of bone marrow cells were present. In no case did there appear to be growth of reticulo-endothelial cells at the expense of the normal blood forming tissue. There was a tendency for the proportion of reticulo-endothelial cells to increase with the duration of the disease but there was a very poor correlation between the proportion of these cells and the severity

of the anemia, leukopenia and thrombocytopenia. Leukopenia appears to precede any great growth of reticulo-endothelial cells in the marrow (patients nos. 4,

TABLE 3 *Sternal Marrow Studies*

Patient	Reticulo endothelial*	R-E Cells Parasitized†	Myeloblasts	Promyelocytes	Neutrophilic Myelocytes	Eosinophilic Myelocytes	Basophilic Myelocytes	Metamyelocytes	PMN Neutrophils	PMN Eosinophils	PMN Basophils	Lymphocytes	Monocytes	Plasma Cells	Pronormoblasts	Basophilic Normoblasts	Polychromatic Normoblasts	Orthochromic Normoblasts	Mitotic Cells	Leukocyte-Erythroid Ratio	Megakaryocytes per million Nucleated Cells‡	% Megakaryocytes with Platelets	
Norm Range	0-2	0-0	2-0	5-0	12-0	1-5	0-3	22-0	20-0	2-0	0-2	10-0	2-0	0-4	4-0		18		1-5	4-5	142	75	
1	2-2	0-0	3-5	1-8	5-19	5-3	0-5	13-32	7-30	5-1	0-7	3-17	5-5	0-2	1-8		7-32		5-2	4-5	85-200	55-87	
2	25	30	0-9	3-0	15-2	0-1	0-0	16-6	3-8	0-0	0-0	20-0	2-8	2-4	0-5	3-8	25-5	4-5	0-9	1-9	36	33	
3	25	10	0-8	2-6	13-9	0-4	0-2	11-0	7-0	0-0	0-0	28-2	1-5	2-4	0-6	4-2	19-4	7-4	0-4	2-2	62	21	
5	5	—	1-0	0-6	2-5	14-3	0-5	0-0	24-0	8-2	0-6	0-2	9-1	3-1	3-0	0-2	3-3	27-6	1-8	1-0	2-0	94	29
6	20	2	1-6	3-0	16-0	0-0	0-0	21-0	5-0	0-0	0-6	7-1	3-5	2-9	2-8	6-8	20-1	8-3	1-3	1-6	62	40	
7	10	—	1-2	2-9	18-5	0-0	0-0	16-7	5-1	0-0	0-0	8-2	1-7	2-2	1-6	9-4	26-3	5-9	0-3	1-3	206	12	
8	40	10	1-6	4-3	13-5	2-9	0-0	12-0	4-8	4-0	0-0	12-0	5-0	6-2	4-3	9-0	15-0	5-0	0-4	2-0	136	2	
9	40	30	0-5	3-4	14-3	0-0	0-0	18-0	5-5	0-0	0-0	7-3	3-7	2-9	2-2	3-9	21-4	16-2	0-7	1-3	76	2	
10	20	—	2-2	3-8	11-4	0-8	0-4	20-0	7-8	0-6	0-0	17-6	1-2	2-2	0-6	3-2	14-4	12-0	1-8	2-3	148	64	
11	20	—	1-0	5-3	9-2	0-9	0-1	19-1	5-5	0-0	0-0	7-3	4-0	2-9	1-4	3-6	28-2	12-7	0-7	1-2	200	14	
15	5	—	1-4	2-6	11-2	1-6	0-2	34-4	7-0	0-6	0-0	13-0	2-8	0-8	0-8	4-6	12-4	6-0	0-6	3-2	150	59	
19	50	—	1-0	3-4	20-4	0-3	0-0	19-3	2-6	0-0	0-0	14-4	2-6	2-6	0-8	1-3	22-2	8-6	0-5	2-0	68	16	
22	15	20	0-6	1-5	18-7	0-8	0-0	21-6	6-6	0-0	0-0	13-6	1-5	5-8	1-5	2-3	18-6	5-5	1-4	2-6	50	23	
23	25	30	0-9	3-0	19-8	0-4	0-0	18-3	2-1	0-0	0-0	4-6	1-5	1-7	1-7	7-8	24-2	12-8	1-2	1-2	90	22	
24	15	—	1-3	3-6	16-0	0-4	0-0	30-4	7-1	0-0	0-0	15-7	3-6	2-6	0-7	2-9	11-7	3-3	0-7	4-4	189	83	
25	15	—	1-8	3-3	10-5	0-0	0-2	27-7	4-2	0-0	0-0	9-4	1-4	1-8	1-3	3-9	22-3	10-4	1-8	1-6	156	5	
26	40	5	0-6	4-2	9-7	0-0	0-0	6-6	3-1	0-0	0-0	11-9	1-7	2-9	1-6	7-7	35-3	13-1	1-6	0-7	183	33	
27	10	30	0-7	2-4	8-8	0-1	0-0	14-4	5-4	0-0	0-0	17-5	2-3	4-0	0-3	6-2	26-2	11-0	0-7	1-3	106	41	
27	25	10	0-2	1-6	12-4	1-4	0-2	30-2	7-0	0-9	0-0	11-3	0-9	1-5	0-5	3-6	22-5	4-5	1-3	2-2	185	73	
Aver	22	10	1-0	3-0	14-1	0-6	0-1	20-1	5-4	0-4	0-0	12-7	2-5	2-8	1-3	4-9	21-8	8-3	1-0	1-8	122	32	

## Patients with complications

4	6	—	0-8	2-0	17-6	0-0	0-0	23-7	4-7	0-2	0-0	14-2	5-1	2-4	0-6	2-4	23-1	2-8	0-4	2-5	82	19
12	5	—	0-6	6-4	42-0	0-0	0-4	17-6	6-6	0-0	0-0	15-6	4-4	1-0	0-0	3-8	0-6	0-8	0-2	18-2	51	23
13	10	20	0-8	3-4	21-2	2-0	0-0	15-0	1-8	0-0	0-0	11-2	4-0	3-0	2-0	3-4	24-6	7-0	0-6	1-7	75	5
14	10	20	0-4	3-6	26-1	0-2	0-4	16-1	1-6	0-0	0-0	8-7	4-7	2-4	0-7	2-0	20-8	11-4	0-9	1-9	65	10
16	47	15	1-1	4-2	21-7	0-0	0-0	22-0	4-7	0-0	0-0	15-2	3-0	2-2	1-5	5-4	13-9	4-5	0-6	3-0	48	15
17	10	—	0-4	3-5	22-3	1-1	0-0	21-9	5-5	0-6	0-0	11-7	2-4	4-6	0-8	3-3	18-7	2-8	0-4	2-9	79	8
18	8	—	0-6	3-0	22-6	0-1	0-0	25-8	10-0	0-8	0-0	14-2	3-1	4-6	0-8	1-2	12-2	0-6	0-4	5-8	112	21
20	20	60	0-4	1-2	17-8	1-4	0-0	28-8	7-0	0-6	0-0	7-8	1-8	3-2	0-6	2-0	19-0	7-6	0-8	2-4	72	27
21	5	—	0-3	2-7	31-5	0-0	0-0	20-0	3-3	0-0	0-0	27-3	6-7	2-4	0-3	0-3	2-8	0-9	1-5	22-3	69	15

Figures given are in per cent except where stated otherwise

\* Estimated and not included in the bone marrow differential calculations

† Estimated

‡ Not included in the bone marrow differential calculation

— Less than

11, 12, 21, 26) Photomicrographs of parasitized reticulo-endothelial cells are presented in figure 7

3 *Leukocytes* The most striking alteration of the leukocytes was in the polymorphonuclear neutrophils. These cells were reduced to less than 8 per cent from the normal of about 20 per cent. This reduction appeared to take place early. The

average value for the 6 uncomplicated cases of approximately one month's duration was 6.4 per cent whereas the average value for the group was 5.4 per cent. This diminution in the adult neutrophils appears to be the first change to take place in the bone marrow. This coincides with the finding of leukopenia in the peripheral blood early in the course of the disease. The percentage of myeloblasts, promyelocytes, neutrophilic myelocytes and metamyelocytes was not significantly altered in most patients, although the metamyelocytes as well as the adult neutrophils were

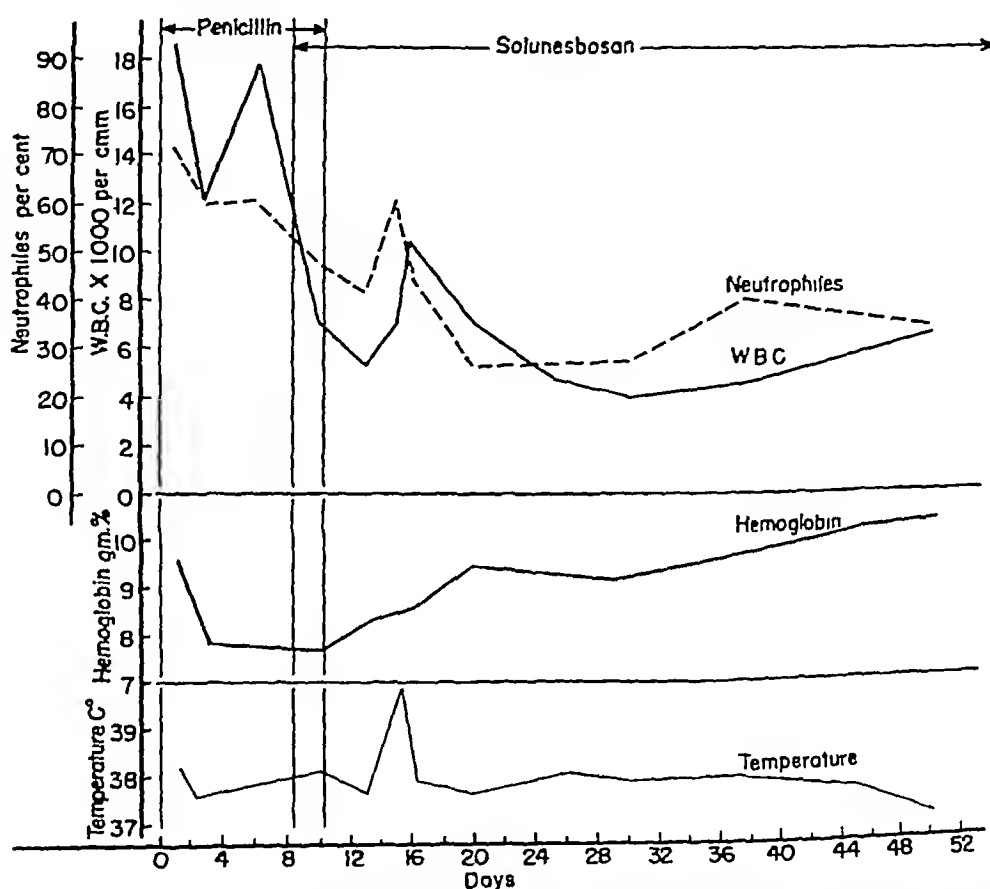


FIG. 6 Patient (no. 18) with kala-azar and severe noma associated with leukocytosis and neutrophilia. Note the development of leukopenia and neutropenia following treatment of the noma with penicillin (1.25 million units intramuscularly) and the return to normal with specific antileishmanial therapy.

markedly diminished in the one case of extremely long duration (no. 25). The eosinophilic myelocytes and adult eosinophils were reduced in numbers, especially in the late cases where they were rarely if at all encountered during the examination of 500 to 1000 cells. Basophilic cells were more infrequently seen than normally. Lymphocytes and monocytes were present in normal numbers. The granulocyte-nongranulocyte ratio was reduced from approximately 5:1 to 1:6:1. The proportion of plasma cells was increased from 0.4 per cent to an average of 2.8 per cent.

4. *Erythroid Cells* Erythroid cells were numerous in the bone marrow in kala-azar, constituting about 36 per cent (normal, 22 per cent) of all cells, exclusive of

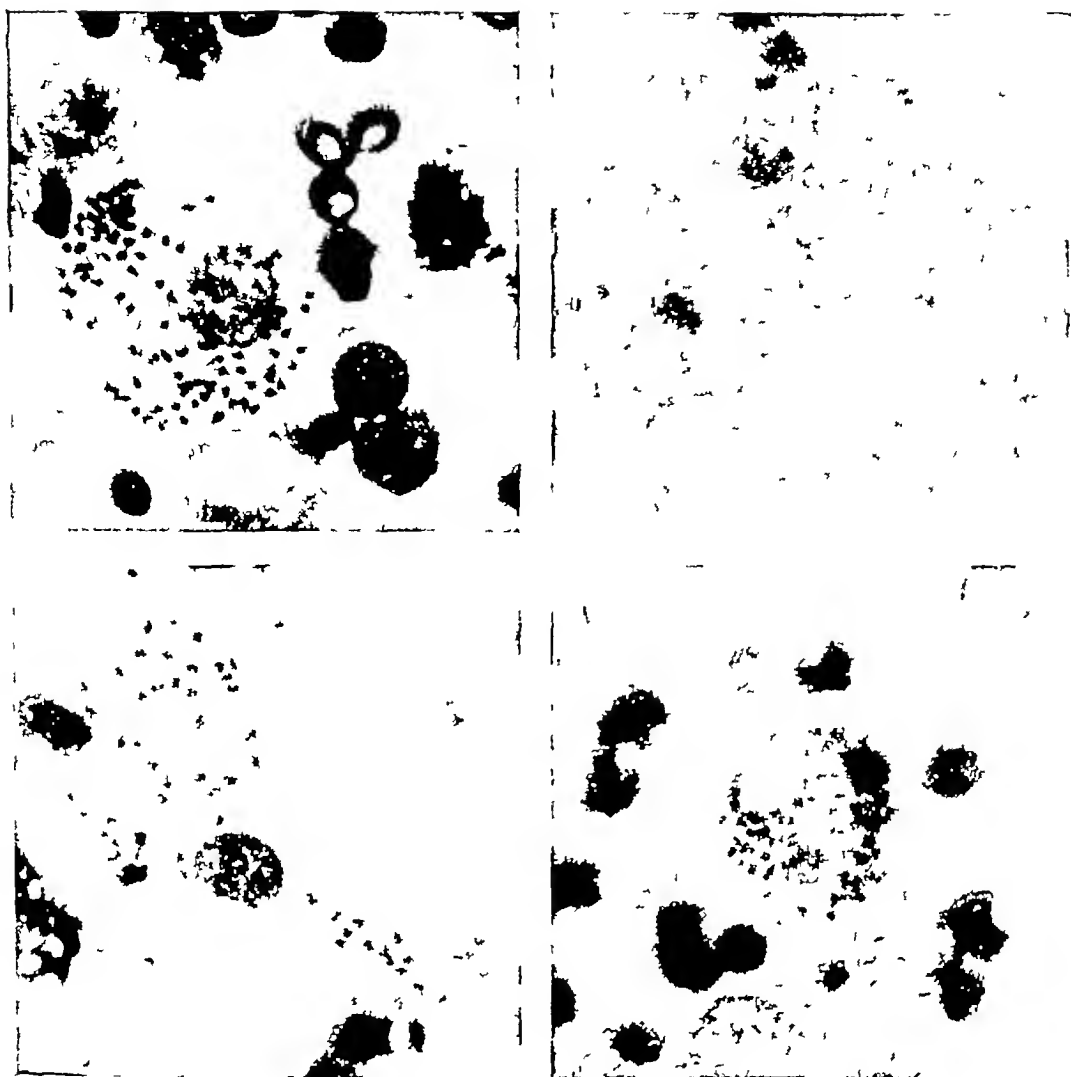


FIG 7 Showing parasitized reticulo-endothelial cells in the sternal marrow

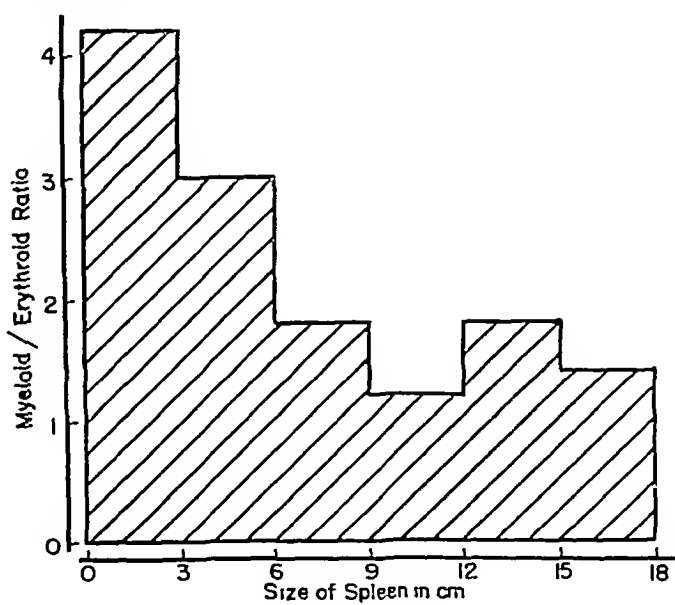


FIG 8 Showing the reduction in the myeloid (leukocyte)-erythroid ratio with progressive enlargement of the spleen



reticulo-endothelial cells. The majority of nucleated red cells were polychromatic normoblasts. Megaloblasts were not encountered and there was no increase in early forms of nucleated red cells. The leukocyte-erythroid ratio was shifted from the normal of approximately 3:5:1 to an average of 1:8:1 (table 3). This ratio decreased as the size of the spleen increased, as shown in figure 8. The granulocyte-erythroid ratio was diminished from 3:1 to 1:2:1.

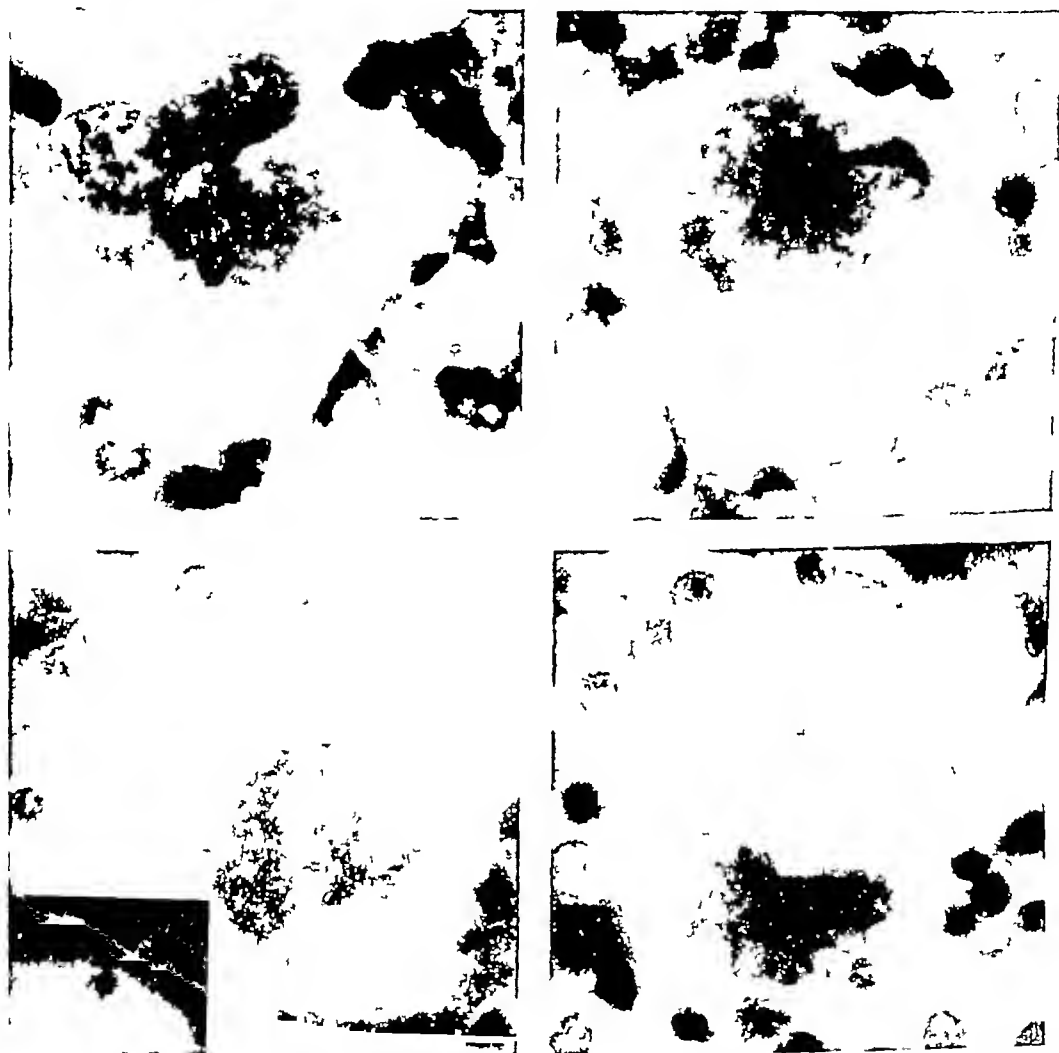


FIG. 9. Typical megakaryocytes from the bone marrow in untreated kala-azar. Note the absence of platelet formation around the periphery of the cytoplasm of the cells.

5 *Megakaryocytes*. The relative number of megakaryocytes in the sternal marrow of the uncomplicated cases varied considerably. The number was less than normal in 6, normal in 11 and slightly greater than normal in one. The number of megakaryocytes per million nucleated cells (including reticulo-endothelial cells) for the entire group of uncomplicated cases averaged 122, as compared with the normal of 142. In view of the fact that the reticulo-endothelial cells were increased in numbers, the ratio of megakaryocytes to nucleated blood forming cells was somewhat higher. The relative number of megakaryocytes in the 9

patients complicated by other diseases was less than normal in 8 and normal in only one

Staining abnormalities were noted in many of the adult megakaryocytes. Their cytoplasm was less granular and took on a purplish hue. The nuclei of many of these cells appeared somewhat degenerated. No striking abnormalities were noted in the differential counts of the megakaryocytes although this was

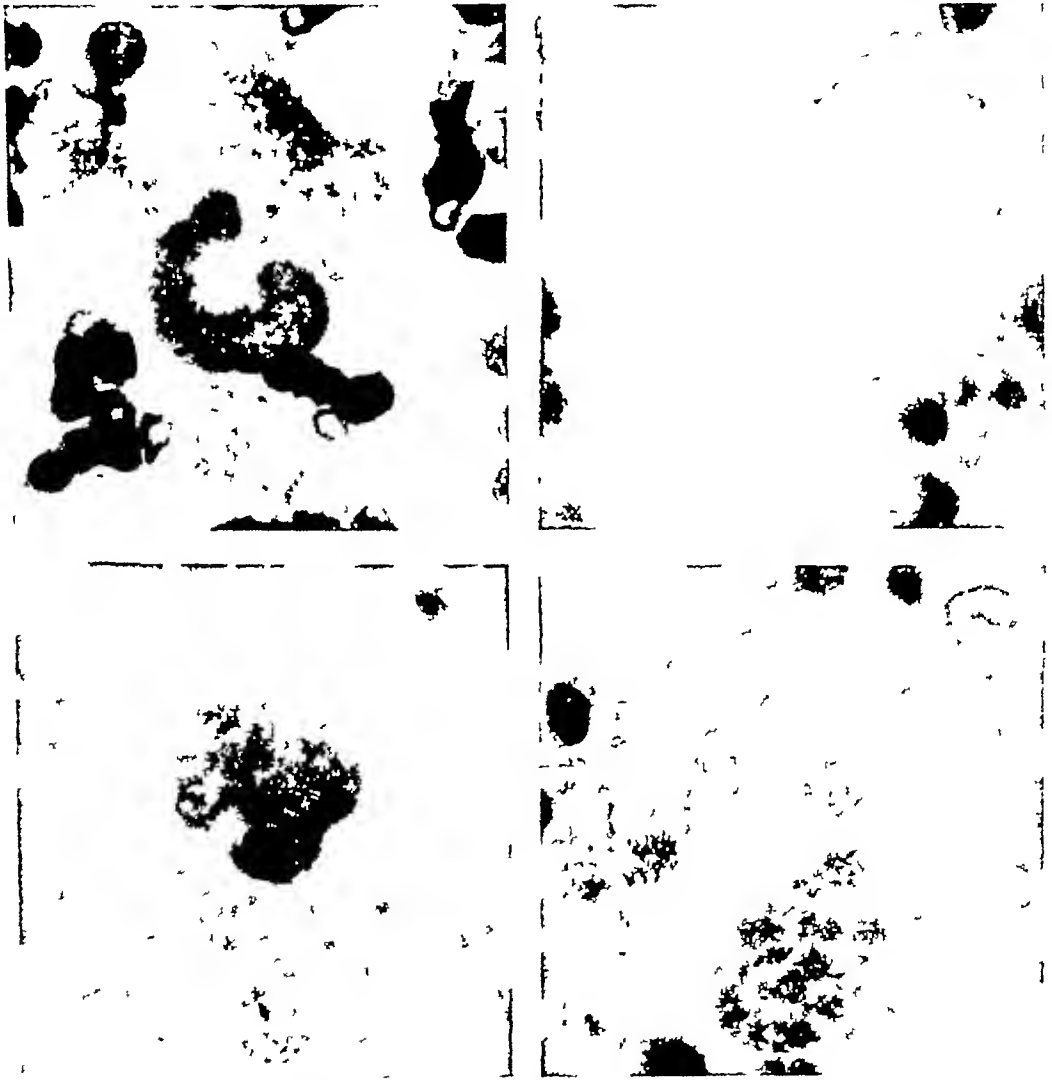


Fig. 10. Typical megakaryocytes from normal sternal marrow. Note the moderate platelet formation around the periphery of the cells.

not studied in detail. All forms from megakaryoblasts through adult megakaryocytes were present. The latter were more frequently seen than any other form.

Platelet production from the megakaryocytes was found to be markedly reduced. In normal marrow approximately 75 per cent (55 to 87) of the megakaryocytes were found to contain platelets or platelet-like bodies at the peripheries of their cytoplasm, whereas in kala-azar platelet production was obvious in only about 32 per cent and then never to any great extent (fig. 9). In normal marrow as many as 60 or more platelets could be seen attached to the periphery of the cytoplasm of

the megakaryocytes and it was not unusual to find between 10 and 50 (fig 10). In the kala-azar marrows there generally were less than 10 platelets surrounding the relatively few platelet-producing megakaryocytes. In only 4 of the marrows was platelet production from megakaryocytes normal and all of these were ex-

TABLE 4 *Blood and Sternal Marrow Studies Following Specific Therapy*

Patient		Blood				Bone marrow																																							
		Days After Onset Therapy	Hemoglobin Gm per cent	White Cell Count per c mm	Platelets in Thousands per c mm	Size of Spleen in C M	Reticulo endothelial	% R E Cells Parasitized	Myeloblasts	Promyelocytes	Neutrophilic Myelocytes	Eosinophilic Myelocytes	Basophilic Myelocytes	Metamyelocytes	PMN Neutrophils	PMN Eosinophils	PMN Basophils	Lymphocytes	Monocytes	Plasma Cells	Promonoblasts	Basophilic Normoblasts	Poly chromic Normoblasts	Orthochromic Normoblasts	Mitotic Cells	Leukocyte/Erythroid Ratio	Megakaryocytes per 1 million Nucleated Cells	% Megakaryocytes with Platelets																	
4	0	11	0	2500	187	7	6	1	0	8	2	0	17	6	0	0	0	23	7	4	7	0	2	0	14	2	5	1	2	4	0	6	2	4	23	1	2	8	0	4	2	4	82	19	
	51	15	2	8300		5	4	0	1	2	0	4	4	0	1	2	0	10	8	19	2	1	2	0	40	8	8	4	2	0	0	0	0	0	2	4	8	0	0	4	8	6	200	86	
	60	15	1	11600	215	5	4	0	1	9	2	1	6	8	1	9	0	4	13	3	18	6	1	9	0	30	4	2	2	1	1	0	0	0	8	5	3	12	5	0	8	4	4	153	79
5	0	11	8	4000	105	12	20	2	1	6	3	0	16	0	0	0	0	21	0	5	0	0	0	0	7	1	3	5	2	9	2	8	6	8	20	1	8	3	1	3	1	6	62	40	
	32	14	8	8700	159	11	5	0	0	3	1	3	16	7	8	7	1	0	28	7	14	8	0	3	0	15	3	1	6	1	0	0	3	1	0	6	0	3	0	0	8	7	100	43	
	46	15	1	10800	208	11	5	0	0	7	0	7	10	0	5	9	0	7	29	6	13	5	2	2	0	16	0	4	1	0	7	0	0	0	0	4	4	11	5	0	0	5	3	140	60
6	0	9	4	2750	175	6	10	1	1	2	2	9	18	5	0	0	0	16	7	5	1	0	0	0	8	2	1	7	2	2	1	6	9	4	26	3	5	9	0	3	1	3	206	12	
	39	12	5	6000	180	4	6	0	0	3	1	4	11	2	8	0	0	15	0	6	7	1	6	0	9	2	4	3	0	6	0	6	1	4	35	7	3	7	0	3	1	4	205	86	
	49	14	5	9800	280	1	5	0	1	6	2	3	8	6	2	7	0	0	16	4	18	0	5	0	0	12	8	5	0	0	8	0	0	0	0	12	4	12	8	1	2	3	0	140	80
9	0	9	4	4100	235	4	20	1	2	2	3	8	11	4	0	8	0	4	20	0	7	8	0	6	0	17	6	1	2	2	2	0	6	3	2	14	4	12	0	1	8	2	4	148	64
	26	16	4	7800	280	3	5	0	0	3	0	6	3	1	3	1	0	3	10	1	17	1	0	6	0	17	9	2	8	0	8	0	6	2	2	21	0	18	7	0	8	1	3	239	78
	36	15	6	9200	280	3	3	0	0	8	0	0	4	7	4	7	0	0	14	5	20	5	1	6	0	19	1	2	4	0	4	0	0	1	2	9	0	21	1	0	2	2	200	83	
14	0	5	0	6500	140	7	10	20	0	4	3	6	26	1	0	2	0	4	16	1	1	6	0	0	0	8	7	4	7	2	4	0	7	2	0	20	8	11	4	0	9	1	9	65	10
	37	13	8	11200		3	5	0	2	6	1	4	8	0	1	4	0	0	35	1	8	0	0	0	0	13	1	4	5	1	1	0	6	3	4	10	6	9	4	0	8	3	2	58	14
	48	15	2	12500	132	2	5	0	0	9	2	1	8	7	9	6	0	9	19	0	17	4	1	8	0	23	3	2	1	0	0	0	0	5	6	2	7	5	0	0	6	3	102	12	
15	0	10	2	1900	122	11	50	1	1	0	3	4	20	4	0	3	0	0	19	3	2	6	0	0	0	14	4	2	6	2	6	0	8	1	3	22	2	8	6	0	5	2	0	68	16
	38	11	8	8500	108	4	30	0	0	6	1	7	8	8	1	3	0	0	22	3	20	3	0	0	0	13	3	1	7	0	6	0	0	0	3	9	7	18	0	1	4	2	6	180	65
	0	4	7	3700	34	13	10	20	0	8	3	4	21	2	2	0	0	0	15	0	1	8	0	0	0	11	2	4	0	3	0	2	0	3	4	24	6	7	0	0	6	1	7	75	5
13	35	8	7	4100		13	8	0	1	2	1	4	8	2	0	3	0	0	20	5	13	5	0	2	0	12	8	2	5	2	0	0	5	3	5	13	7	18	5	1	2	1	8	82	12
	46	9	7	6100	35	13	5	0	1	3	2	4	8	4	1	8	0	0	17	6	11	8	0	9	0	16	3	1	8	1	6	2	0	2	9	13	5	16	8	0	9	1	8	132	55
	0	9	6	18800	144	9	8	1	0	6	3	0	22	6	0	1	0	0	25	8	10	0	0	8	0	14	2	3	1	4	6	0	8	1	2	12	2	0	6	0	4	5	8	112	21
18	33	9	6	4500	130	7	8	0	1	4	2	4	4	3	3	4	0	3	23	7	16	6	0	0	0	13	3	1	6	2	7	0	3	3	0	12	0	14	3	0	7	2	4	167	28
	0	9	0	2350	65	13	40	10	1	6	4	3	13	5	2	9	0	0	12	0	4	8	4	0	0	12	0	5	0	6	2	4	3	9	0	15	0	5	0	0	4	2	0	136	2
	66	7	3	4400	18	13	30	0	1	1	1	1	4	8	0	2	0	0	22	0	12	2	0	2	0	8	2	3	2	4	3	1	0	1	9	19	4	18	9	1	5	1	4	194	8
16	85	7	4	4000	25	13	30	0	1	8	2	0	8	6	1	0	0	0	26	0	2	0	0	2	0	6	6	1	2	2	2	0	8	3	6	27	2	16	2	0	6	1	1	225	82
	0	9	0	8600	113	4	47	15	1	1	4	2	21	7	0	0	0	0	22	0	4	7	0	0	0	15	2	3	0	2	2	1	5	5	4	13	9	4	5	0	6	3	0	-48	15
	37	7	4	3350	104	5	40	1	0	7	3	0	14	0	1	3	0	0	10	7	5	3	0	3	0	22	1	3	0	0	3	1	3	7	4	23	3	5	6	1	7	1	6	98	30
20	0	7	7	6300	158	15	20	60	0	4	1	2	17	8	1	4	0	0	28	8	7	0	0	6	0	7	8	1	8	3	2	0	6	2	0	19	0	7	6	0	8	2	4	72	27
	29	9	3	4700	137	15	20	30	1	5	1	6	5	9	3	1	0	3	15	6	8	7	0	9	0	17	8	3	4	6	2	1	9	5	3	20	9	5	0	1	9	2	0	123	66
	0	6	0	2950	110	6	40	30	0	5	1	4	16	3	0	0	0	0	20	0	5	5	0	0	0	7	3	3	7	0	9	2	2	3	9	21	4	16	2	0	7	1	3	76	2
8	64	7	2	3200	51	6	25	2	0	3	0	6	8	3	0	3	0	0	9	8	1	8	0	0	0	12	8	1	8	1	3	0	3	1	8	4	1	6	19	3	0	0	6	32	1
	70	5	6	1900	102	5	25	1	0	0	0	3	2	8	0	0	0	0	4	3	4	0	0	0	0	12	0	2	3	0	9	1	7	5	7	46	2	17	0	2	8	0	4	80	10
	88	6	4	4100	114	7	25	0	0	4	1	8	9	0	0	6	0	0	13	0	1	8	0	0	0	6	2	0	6	0	4	1	4	8	2	43	2	12	8	0	6	0	5	168	49
	101	6	3	2400	124	7	25	0	1	0	1	8	13	0	0	6	0	0	28	0	3	0	0	0	0	6	8	1	8	0	8	0	8	4	4	31	0	6	2	0	8	1	4	100	31

tremely early cases (nos 9, 11, 23, 27) Free platelets were scarce in the bone marrow preparations. Large groups of platelets, such as are noted in normal marrow, were almost never seen.

6 *Changes in the Sternal Marrow During Therapy* Follow-up studies were available in 12 patients under treatment. These studies are presented in table 4. Due to

existing circumstances a variety of antileishmanial drugs, American, German, British, and Japanese, was used. Some of these were more efficacious than others. The response of 8 of the patients (nos. 4-6, 9, 13-15, 18) was good, in 4 (nos. 7, 8, 16, 20) the response was poor. The changes in the peripheral blood of one patient (no. 18) during treatment are presented in figure 6.

During effective therapy parasitized reticulo-endothelial cells rapidly disappeared and the percentage of reticulo-endothelial cells gradually diminished. There was a "shift" of the leukocytes of the bone marrow to the right. The proportion of neutrophilic myelocytes diminished and the polymorphonuclear neutrophils increased. The metamyelocytes temporarily increased, decreased or remained unchanged. At the same time there was a significant increase in eosinophilic myelocytes and adult eosinophils. Basophilic myelocytes were more frequently seen. Lymphocytes became more numerous. The relative number of plasma cells diminished to normal. Nucleated red cells became less numerous as the leukocyte-erythroid ratio increased to normal. As this change took place the proportion of polychromatic normoblasts to orthochromatic normoblasts decreased from 2.9 to 0.7. These changes are shown graphically in figure 11.

The number of megakaryocytes per million nucleated cells increased during effective therapy. The only exception to this was patient no. 6. In this patient the number of megakaryocytes per million nucleated cells was slightly greater than normal before therapy and the number decreased to within the normal range during therapy.

The percentage of megakaryocytes forming platelets increased to within the normal range in 6 of the 8 patients responding to therapy (table 4). Not only did the percentage of megakaryocytes forming platelets increase but the average number of platelets attached to each megakaryocyte was greatly increased, even beyond the normal. This is illustrated in figure 12. The number of platelets attached to each of 100 megakaryocytes in the marrow of the same patient (no. 6) was counted as accurately as possible before and after treatment and compared with a normal.

Photomicrographs of megakaryocytes taken from the same preparations as used in preparing figure 12 are presented in figures 9, 10, and 13. These photographs again illustrate the absence of platelet formation in untreated kala-azar (fig. 9) and the greatly increased platelet formation in the marrow following treatment (fig. 13) as compared with the normal (fig. 10).

Following the production of platelets by the megakaryocytes, huge groups of platelets were frequently seen in the marrow smears. These were larger and more numerous than those seen in smears made from normal marrow and were in sharp contrast to the very few platelets seen scattered about the marrow in the preparations made from untreated cases. The platelet count in the peripheral blood then approached normal as a rule, although no increase was noted in patients 13 and 15. However, the platelet counts were just at the lower limit of normal and it would be expected that the platelet rise in the peripheral blood would occur later than the bone marrow change.

Two patients (nos. 14, 18), in whom there was no increase in platelet production

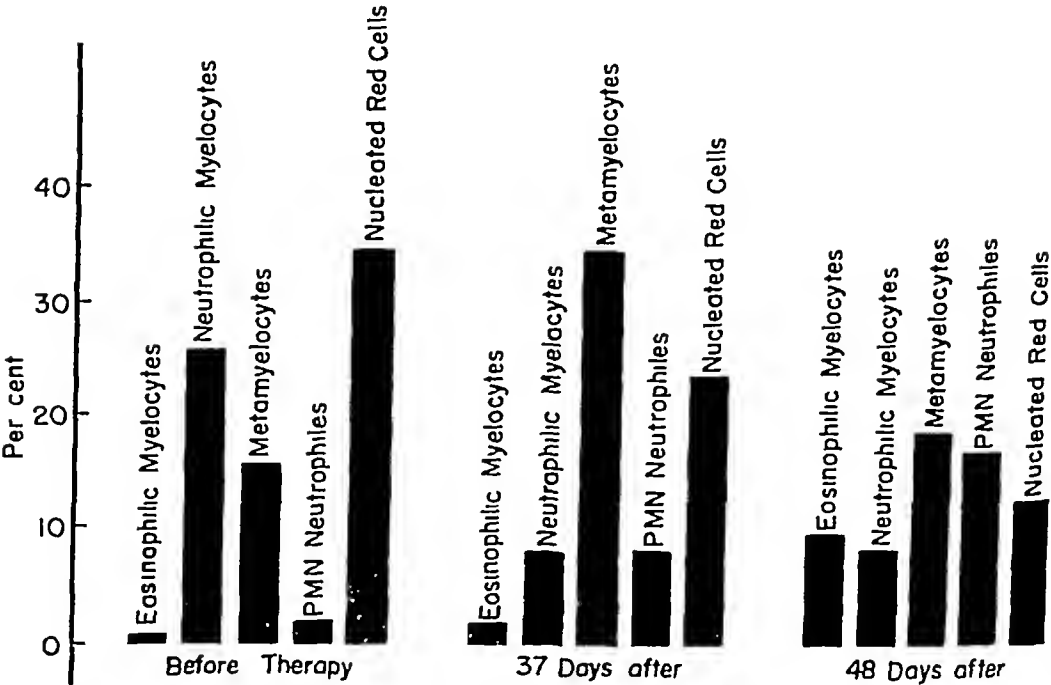


FIG 11 Bone marrow changes in a patient (no. 14) with kala-azar before and during specific anti-leishmanial therapy (solunesbosan)

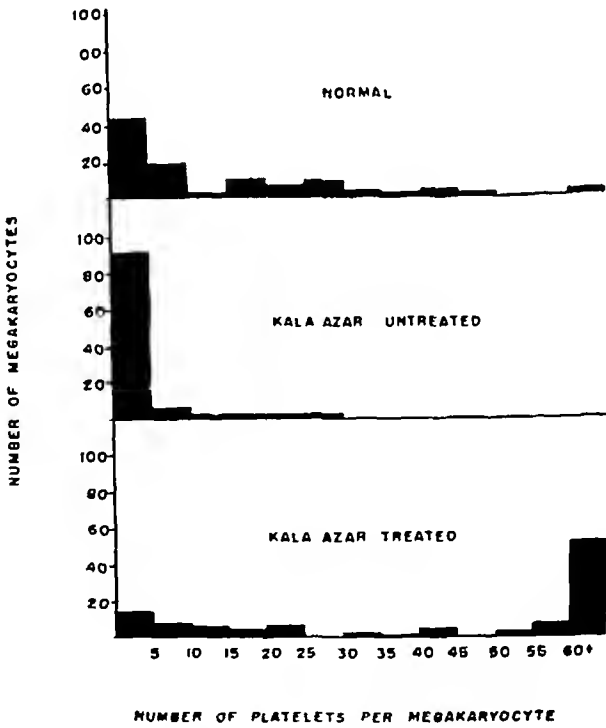


FIG 12 Number of platelets attached to the cytoplasm of 100 megakaryocytes in normal marrow, in a patient (no. 16) with kala-azar prior to specific therapy and in the same patient after specific therapy

in the bone marrow, were both complicated in that severe infections existed. As expected, no rise in the platelets in the peripheral blood was noted.

From table 4 it is evident that the first change which took place in the platelet forming tissue was an increase in the number of megakaryocytes. Following this,

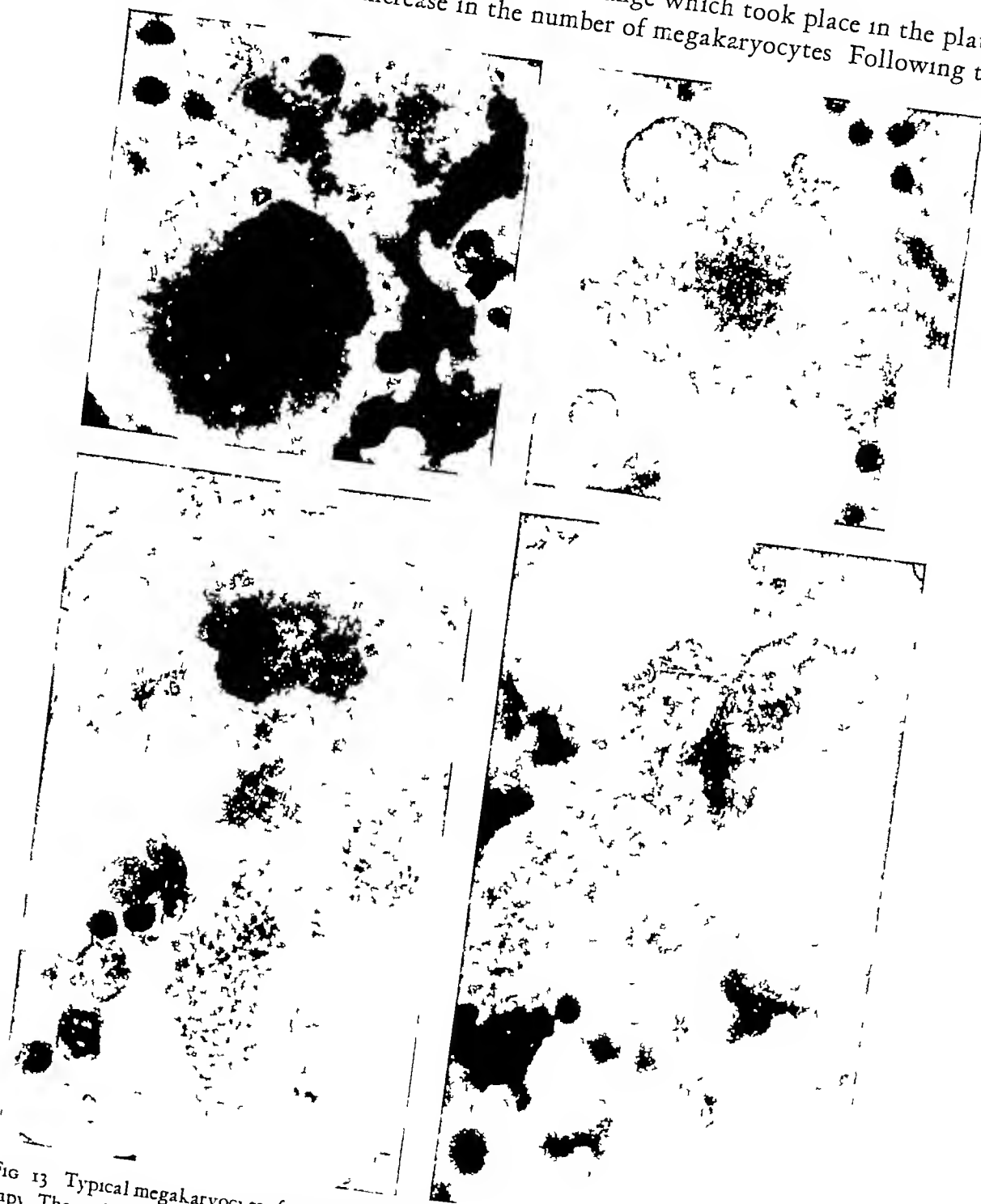


FIG. 13. Typical megakaryocytes from the bone marrow of a patient with kala-azar following specific therapy. These photomicrographs were taken from a preparation of sternal marrow taken from the same patient (no. 6) as were those in figure 9. Note the large number of platelets at the periphery of the cells. Platelet production from the megakaryocytes increased and later there was a rise in the number of platelets in the peripheral blood. This took place after there had

been a significant rise in the number of leukocytes and the quantity of hemoglobin in the blood. Thus the three cellular elements of the blood were restored to normal in the same order as their reductions from normal had taken place. The increase in platelets in the peripheral blood was not as dramatically rapid as occurs following splenectomy in primary thrombocytopenic purpura. This is natural since the reduction in the size of the spleen in kala-azar in response to therapy is very gradual over the course of months.

Four patients (nos 7, 8, 16, 20) responded poorly to therapy (table 4). There was no detectable clinical improvement, no diminution in the size of the spleen, and little or no change in the peripheral blood. *Leishmania* were present in the bone marrows twenty-nine days after treatment was commenced in one patient (no 20), after thirty-seven days in another (no 16) and after seventy days in another (no 8). *Leishmania* were found in the spleen in the last named patient on the one hundredth day of therapy. The marrows remained normoblastic and a 'shift' to the right in the myeloid series did not occur. There was, however, an increase in the number of megakaryocytes per million nucleated cells in each case and in 2 of the patients (nos 7, 20) platelet production by the megakaryocytes had commenced. Three of the patients (nos 7, 8, 16) were treated initially with a Japanese preparation, solunesbosan. Two of the patients (nos 16, 20) had complicating infections and 2 (nos 8, 16) developed agranulocytosis during therapy. All 4 of these patients were heavily parasitized at the onset of therapy and none were in the early stage of the disease. Patient no 20 was given 50 mg of pteroyl-glutamic acid orally daily for 10 days without any apparent effect on the leukopenia.

#### DISCUSSION

Differential counts on the sternal marrow from cases of kala-azar have thus revealed a rather characteristic pattern. Myeloblasts, promyelocytes, neutrophilic myelocytes and metamyelocytes were present in approximately normal proportions but polymorphonuclear neutrophils were markedly reduced and eosinophilic cells were rarely seen. In the patient with disease of two years duration, the metamyelocytes were also reduced in number and in one patient with kala-azar and advanced bilateral pulmonary tuberculosis approximately 62 per cent of the myeloid cells were myelocytes or younger forms even though the leucocyte-erythroid ratio was 18:1. This would seem to indicate either an inability of the leucocytes to mature fully or rapid dissolution of the adult cells. Study of the bone marrow has shown, however, that this defect can, under certain circumstances, be overcome since occasionally patients with secondary bacterial infections develop leucocytosis in spite of the existence of severe kala-azar with marked granulocytopenia.

The presence of staining abnormalities, the reduction in total numbers of megakaryocytes in some cases, and the absence of a normal degree of platelet production suggest an abnormality in the development of the megakaryocytes which results in a failure to produce platelets. This offers an explanation for the thrombocytopenia seen in kala-azar. If platelets were being destroyed rapidly in the peripheral blood one would expect to find hypertrophy of the megakaryocytic

tissue in the marrow with each megakaryocyte producing a maximal number of platelets

Nucleated red cells, especially polychromatophilic normoblasts were numerous in the marrow. It is impossible to say from our data whether the increase was absolute or only relative to a decrease in the leucocytic tissue. One might postulate that since there are many normoblasts in the marrow there is a failure of these cells to make the transition to erythrocytes. Such a situation does exist in the bone marrow in patients with anemia due to iron deficiency and in patients with disturbed hemoglobin synthesis due to lead intoxication. On the other hand, it is also true that in patients with active erythropoiesis, such as occurs in anemia due to hemorrhage or hemolysis, there is an increase in normoblastic activity in the marrow.

None of our patients were jaundiced. Rachmilewitz, Braun and De Vries<sup>38</sup> have reported a single patient with kala-azar, macrocytic anemia, reticulocytosis, normoblastic bone marrow, jaundice and a significantly increased excretion of urobilinogen in the urine and feces. They state "The type of anemia, the hyperplastic bone marrow, the increased excretion of urobilinogen before treatment, and the subsequent changes following treatment strongly suggest increased red cell destruction (most probably by phagocytosis) as the cause of the anemia." From our studies we cannot rule out this possibility but it should be noted that patients with chronic liver disease (cirrhosis) and jaundice may have an increased excretion of both urinary and fecal urobilinogen in the absence of increased hemolysis.<sup>39</sup> The fact that the patient reported by Rachmilewitz, Braun and De Vries had macrocytic anemia and jaundice suggests that their patient varied somewhat from the usual patient with kala-azar.

As pointed out in the introduction it has been generally accepted that the hematologic changes seen in kala-azar are the result of impairment of the hemopoietic function of the bone marrow which is destroyed mechanically by the overgrowth of parasitized reticulo-endothelial cells, that is, the anemia is classed with the myelophthisic or leuko-erythroblastic anemias. However, the hematologic changes in kala-azar differ in several respects from those seen in patients with carcinomatosis, osteosclerosis, multiple myeloma, myelosclerosis and marble bone disease. In such examples of so-called "myelophthisic anemia" the most significant abnormality of the erythrocyte series in the peripheral blood is the presence of nucleated red cells in numbers quite out of proportion to the degree of anemia.<sup>30-35</sup> As many as 53 nucleated red cells per 100 leucocytes have been observed in cases in which there was little anemia.<sup>30</sup> Polychromatophilia and stippling are frequently seen. Myelocytes in the peripheral blood are almost a constant feature.<sup>31, 32</sup> and myeloblasts are occasionally seen. The leukocyte count is usually normal, occasionally increased, and only rarely reduced and the differential white cell formula usually maintains its normal proportions.<sup>30-35</sup> In kala-azar nucleated red cells are rarely seen in the peripheral blood. In our own series of patients they were seen in only 5 patients and then in small numbers. Napier and Sharma<sup>9</sup> and Keefer, Khaw and Yang<sup>5</sup> did not observe nucleated red cells in the peripheral blood of any of their patients even when severe anemia was present.



Kuroya et al.<sup>4</sup> recognized normoblasts in only 17 per cent of their cases and then never in very great numbers. Polychromatophilia and stippling are also rarely seen. A single myelocyte was seen in two smears in the series reported here. Kuroya et al.<sup>4</sup> observed myelocytes in only one case out of 151. In uncomplicated kala-azar there is almost invariably a leukopenia. In the differential white cell formula in kala-azar the lymphocytes, in contrast to what occurs in myelophthisic anemia, predominate.

Vaughan<sup>32, 35</sup> has reported that leuko-erythroblastic anemia (myelophthisic anemia) is associated with an increase rather than a decrease of skeletal red marrow and is not dependent upon mechanical limitation of the marrow. She was able to find little correlation between the degree of mechanical blocking and the degree of anemia. In some of her cases with great degrees of occlusion of the marrow there was little anemia. In the series of patients presented in this study infiltration of the sternal marrow by reticulo-endothelial cells varied from 5 to 50 per cent and there was little correlation between the degree of infiltration and the severity of the blood changes. Leukopenia frequently appeared before there was significant infiltration of the marrow. This has also been observed by others.<sup>24, 26</sup> Furthermore, evidence of an extension of the functioning bone marrow in kala-azar has been observed repeatedly.<sup>11, 19, 20</sup> In normal adults only approximately half of the marrow is in an active state. Thus, if all of the marrow is made available and if 50 per cent of this is invaded by reticulo-endothelial cells there may still be no reduction from the normal in the total amount of functioning tissue.

The hematologic changes in kala-azar are similar in many respects to those seen in Gaucher's disease, Still-Chauffard-Felty syndrome, tuberculous splenomegaly and 'primary splenic pancytopenia'.<sup>30</sup> In these so-called "hypersplenic states" there is anemia with little evidence of blood regeneration, neutropenia with a relative lymphocytosis and thrombocytopenia. Hirschboeck<sup>27</sup> has reported differential counts of sternal marrow smears in two cases of Still-Chauffard-Felty syndrome. In both cases the marrow was hyperplastic and normoblastic. There was a marked shift to the left of the myeloid elements and the majority of the cells were myelocytes and metamyelocytes. Similar findings have been reported by Doan and Wright<sup>25</sup> in 3 cases of 'splenic pancytopenia'. In 2 of these cases the myeloid-erythroid ratio was reversed, 1:4. In Banti's syndrome and in other splenomegalic states Limarzi and his co-workers<sup>37</sup> have reported myeloid hyperplasia in the earliest stage and "maturation arrest" of the myeloid and megakaryocyte tissue later. In the last stage of the condition marked erythroid immaturity was found as well.

In kala-azar the reduction in the numbers of erythrocytes, leukocytes and platelets is, in general, proportional to the degree of splenic enlargement.

One apparent point of difference between the bone marrow in kala-azar and that in symptomatic 'hypersplenic' thrombocytopenic purpura is that Dameshek and Miller<sup>26</sup> in 5 cases of the latter (Gaucher's disease, infectious splenomegaly, cirrhosis of liver, splenic vein thrombosis, and Felty's syndrome) found the number of megakaryocytes increased and a normal proportion of platelet-producing cells. Their results in this group of cases differed from their findings in idiopathic

thrombocytopenic purpura (which they consider a form of hypersplenism) in that the proportion of platelet producing cells was markedly reduced in the idiopathic group \*

Although there is, thus, a certain amount of evidence suggesting that the blood changes associated with kala-azar are due to hyperfunction of the spleen, it is clear that no direct evidence has been given in this paper for the hypersplenic theory. Such evidence could come from the demonstration that the anemia, leukopenia and thrombocytopenia are alleviated by splenectomy †. This was considered but for various reasons was not performed. Experiments with splenectomy are now under way in animals experimentally infected with kala-azar and it is anticipated that this will be the subject of another communication ‡.

### SUMMARY

1. The peripheral blood changes in uncomplicated kala-azar are those of pancytopenia, namely, anemia, leukopenia and thrombocytopenia. The red blood cell morphology is normal and there is very little evidence of increased erythrogenic activity. The leukopenia is due to a reduction in all types of cells, especially neutrophils.

2. When the disease is complicated by other infections the anemia is more severe and anisocytosis, poikilocytosis, and polychromatophilia may appear and normoblasts may occasionally be seen in the peripheral blood. Leukocytosis may develop, the leukopenia may persist or the syndrome of agranulocytosis may intervene.

3. As the duration of the disease increases, the spleen tends to become larger and the anemia, leukopenia and thrombocytopenia become progressively more severe. Leukopenia generally appears first, followed by anemia and finally thrombocytopenia. The degree of leukopenia, anemia and thrombocytopenia follow closely the degree of splenic enlargement.

4. The bone marrow in kala-azar is hyperplastic and infiltrated by reticulo-endothelial cells. In spite of this there appears to be an abundance of blood forming tissue, especially erythropoietic tissue.

5. Differential cell studies on preparations of sternal marrow reveal a marked

\* More recent studies by Dameshek and Estren indicate a very definite diminution in platelet production by megakaryocytes in various types of hypersplenism, both due to known cause (i.e. symptomatic or secondary) and in idiopathic cases, the findings being similar to those in idiopathic thrombocytopenic purpura. *Ed*

† Since this paper was submitted for publication a report has appeared by Burchenal, Powers and Haedicke (*Am J Trop Med* 27: 699, 1947) of a case of leishmaniasis with severe anemia and leukopenia which was refractory to antileishmanial therapy. Following splenectomy there was a rapid and sustained increase in leukocytes and hemoglobin. Reference is made in this paper to two other case reports of leishmaniasis in the literature in which splenectomy was followed by a rapid rise in leukocytes.

‡ Preliminary results indicate that hamsters experimentally infected with *Leishmania donovani* develop a leukopenia (5,625 cu. mm.) as compared with normal noninfected hamster (10,625 cu. mm.). Two months following splenectomy the leukocyte count had risen to 14,437 cu. mm. in the infected group, whereas there was only a slight increase (12,475 cu. mm.) in the normal noninfected group following splenectomy.

reduction in the polymorphonuclear neutrophils and eosinophils Myeloblasts, promyelocytes, neutrophilic myelocytes and metamyelocytes are present in approximately normal proportions as are the lymphocytes and monocytes Plasma cells are somewhat increased Erythroid cells, especially polychromatic normoblasts are numerous and the leukocyte-erythroid ratio is altered, more than the normal proportion of normoblasts being found Megakaryocytes are present in normal or slightly reduced numbers Staining abnormalities are noted in these cells and there is a striking reduction in platelet production

6 During effective anti-leishmanial therapy parasitized reticulo-endothelial cells disappear and the percentage of reticulo-endothelial cells gradually diminishes as the polymorphonuclear neutrophils increase There is a significant increase in the eosinophilic cells Lymphocytes become more numerous and the plasma cells diminish in number Nucleated red cells become less numerous and the leukocyte-erythroid ratio returns to normal At the same time the proportion of orthochromatic normoblasts to polychromatic normoblasts increases The relative number of megakaryocytes increases and platelet formation from the megakaryocytes is accelerated even beyond the normal Huge groups of platelets are frequently seen in the marrow smears A rise in platelets in the peripheral blood takes place late, after there has been a significant rise in hemoglobin and leukocytes The three cellular elements are restored to normal in the peripheral blood in the same order as their reduction from normal

7 Evidence is presented which contradicts the view that the pancytopenia is due to a crowding out of the bone marrow by reticulo-endothelial cells

8 Certain similarities between the hematologic changes in this disease and those accompanying the hypersplenic syndromes are noted

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# THE EFFECTS OF ESTROGENS ON THE BONE MARROW OF ADULT FEMALE DOGS

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ESTROGENS have been found to have profound effects on the hemopoietic system of dogs<sup>1-14</sup>. Large doses of natural or synthetic estrogens produced hemorrhagic purpura, gradual anemia, and a profound leucocytosis which was followed by a leucopenia. Differential counts showed that the neutrophils were the cells responding to the injections, and Arneeth counts showed a swing to the left followed by a corresponding shift to the right. These injections also produced a slight liver damage. Although the time required to produce death varied with each animal, a daily dose of 50 mg. of estrogen ultimately killed the dog.

Except for the work of Arnold and his co-workers, little attention has been given changes in the bone marrow as a result of estrogen injections. Accordingly, this experiment was performed to describe the changes produced by estrogens in the bone marrow of adult female dogs.

## METHODS

Adult female dogs of 8-12 kilograms body weight were used in this study.

Each dog was trained to stay on the dog board and blood was removed from femoral vessels without anesthesia. Erythrocyte and white cell counts were made with U.S. certified pipets and the improved Neubauer counting chamber. Smears were made with the two-coverglass technic and differential counts were made on 200 cells. Hemoglobin determinations were made with a Hellige hemometer.

Bone marrows were studied in the following manner. The whole femur was removed at autopsy. A single  $1\frac{1}{2}$  inch piece was removed at the proximal end of the femur, the proximal cut being made with a saw just distal to the trochanters. The piece of bone was then split lengthwise with bone forceps and the top half of the bone removed leaving the bone marrow intact in a "boat" of bone. This was then placed in Helly's fixative. The bone marrow was removed from the "boat" of bone after it had become firm in the alcohols. After embedding in paraffin, it was sectioned at 6 micra and stained with toluidin blue and eosin. Differential counts were made on 500 cells.

All injections were given intramuscularly and in sesame oil.

## RESULTS

Several blood examinations were performed on each dog before estrogen therapy was given. The average blood figures obtained from 45 separate blood samples from 14 dogs are summarized in table 1.

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This work was done with the aid of a grant from the Committee on Research in Endocrinology of the National Research Council.

\* With the technical assistance of Miss Betsy Nadell and Mr. Salvatore Lunetta.

Figure 1 shows a typical curve for the total white cell count of an adult dog under estrogen treatment. The height of the curve varied with each animal and some dogs exhibited a second curve but the terminal effects were the same. Each dog was autopsied when the leucocyte count was at a level indicated in figure 1.

TABLE 1—Normal blood figures for adult female dogs taken from 45 separate blood samples from 14 dogs  $\pm$  = standard deviation

Erythrocyte count in millions per cu mm	6.55 $\pm$ 0.73
Hemoglobin in Gm per 100 cc	14.8 $\pm$ 2.1
Leucocyte count in millions per cu mm	13.2 $\pm$ 4.8
Neutrophils in per cent	69.6 $\pm$ 9.1
Eosinophils in per cent	4.2 $\pm$ 1.6
Basophils in per cent	0.1 $\pm$ 0.21
Lymphocytes in per cent	19.7 $\pm$ 8.6
Monocytes in per cent	2.8 $\pm$ 2.2

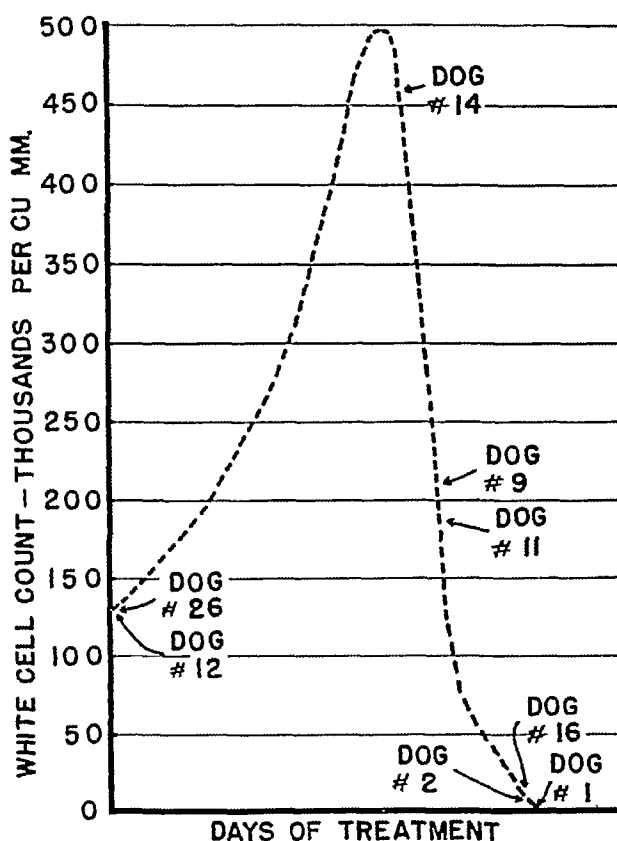


FIG. 1. Showing a typical leucocyte response of adult dogs to injections of large doses of estrogen. The dogs described in this paper were autopsied when the total white counts were as indicated.

This gives a progressive story of the changes produced in the bone marrow during treatment with estrogens.

*Dogs No. 12 and No. 26.* These 2 animals served as controls and were autopsied after no treatment. The bone marrows of the 2 dogs were similar in appearance (figure 2). The myeloid elements were more concentrated toward the periphery and the marrow gave the appearance of a normal distribution of cellular elements.

Differential counts showed that 40.4 per cent of the cells were erythroid in nature and 43.7 per cent of the cells were neutrophilic elements. Further details are given in table 2.

These counts differ slightly from counts reported by other workers. Alexandrov<sup>15</sup> reported 23.4 per cent erythroid elements and 43.0 per cent neutrophilic elements, Stasney and Higgins<sup>16</sup> found 59.0 per cent and 24.2 per cent respectively, Mulligan<sup>17</sup> reported 40.1 per cent erythroid and 50.1 per cent neutrophilic, and Meyer and

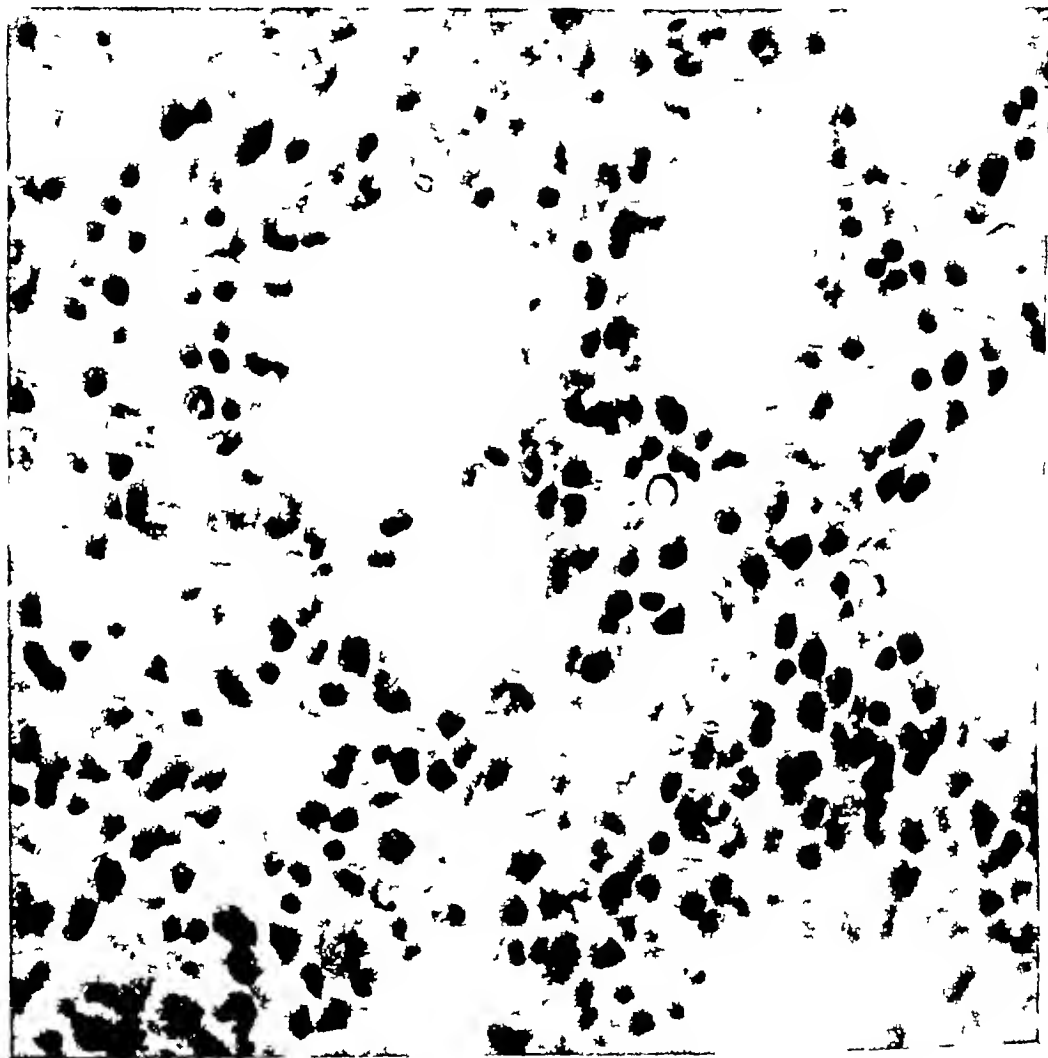


FIG. 2. Bone marrow of a normal control dog. Note the normal distribution of myeloid elements.

Bloom<sup>18</sup> have found erythroid elements to be 38.7 per cent of the bone marrow cells and neutrophilic elements to be 45.7 per cent of marrow cells. These four groups of workers used the sternum, rib, rib, and ilium respectively as a source for marrow while the present work utilized the femur. Stasney and Higgins,<sup>16</sup> however, found that the source of the bone marrow made little difference as far as the distribution of the cells was concerned.

*Dog No. 14.* This animal was autopsied after twenty-five days of treatment with a total of 89.0 mg. of stilbestrol. \* The leucocyte count was close to the peak of rise, having increased from 10.5 thousand cells

\* I am greatly indebted to Dr. C. F. Church of the Squibb Institute for Medical Research for the stilbestrol used in this experiment.

TABLE 2—*Differential counts on bone marrows of adult female dogs treated with estrogen*  
*Cells rarely found have not been included*

Dog	Total leucocyte count in thousands per cu mm	Erythroid elements	Neutrophilic elements	Eosinophilic elements	Promyelocytes	Hemocy toblasts
Normal controls	13 2	%	%	%	%	%
# 14	46 0	40 4	43 7	7 0	3 8	4 7
# 9	21 1	3 6	90 0	0 4	2 6	3 4
# 11	18 8	7 6	86 4	1 4	1 2	3 0
# 16	1 2	31 4	64 8	0 2	0 0	3 2
# 2	0 8	97 4	0 6	0 6	0 0	1 0
# 1	0 2	96 5	0 0	0 5	0 0	3 0
		Not enough cells to count				

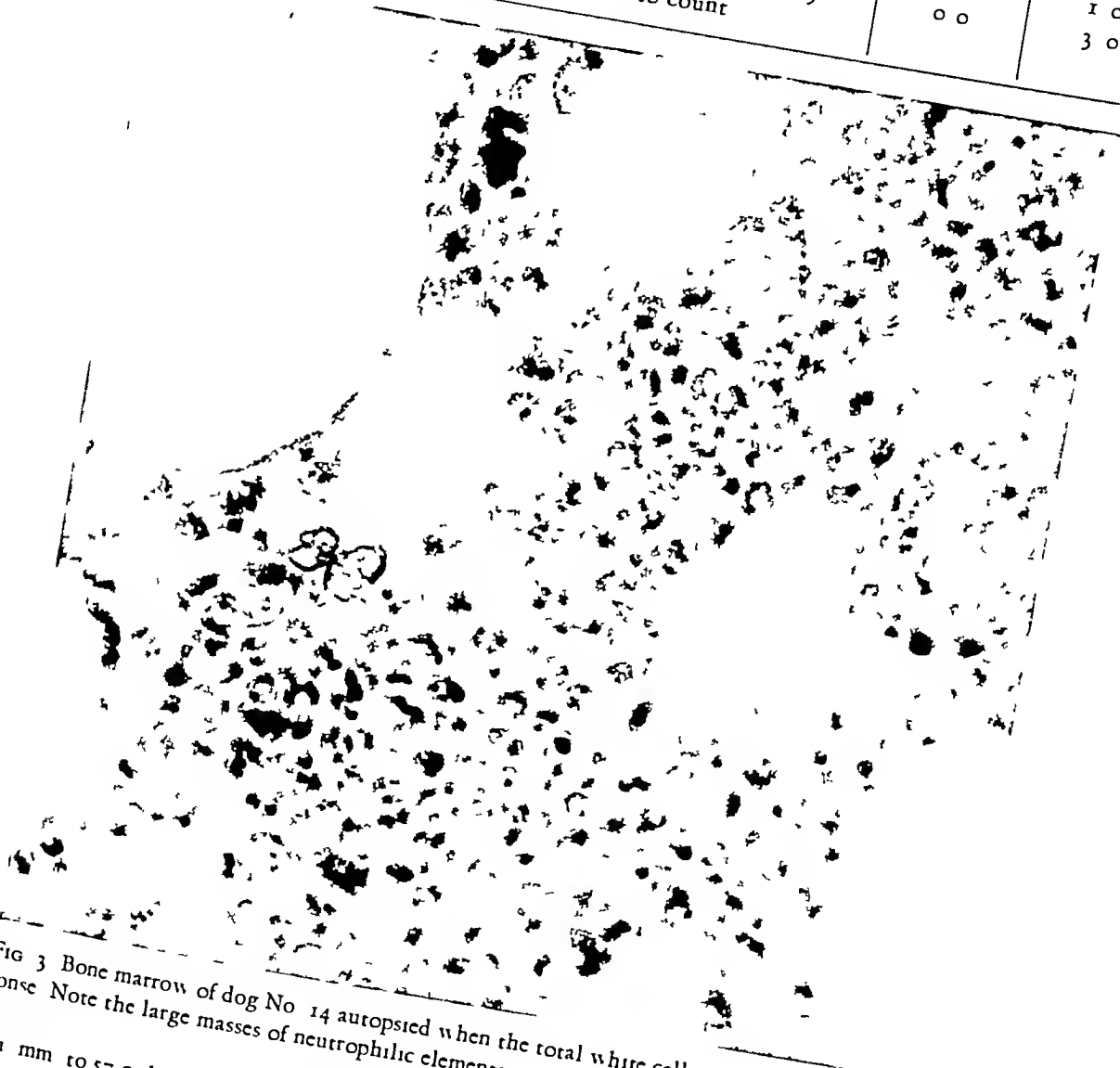


FIG 3 Bone marrow of dog No 14 autopsied when the total white cell count was near its maximum response. Note the large masses of neutrophilic elements per cu mm to 57 0 thousand cells, and then decreased to 46 0 thousand cells per cu mm at the time of autopsy. The erythrocyte count was 7 34 million cells per cu mm, hemoglobin, 18 5 Gm per 100 cc



EFFECTS OF ESTROGENS ON BONE MARROW OF DOGS

neutrophils, 91.0 per cent, eosinophils, 2.0 per cent, basophils, 1.0 per cent, lymphocytes, 5.5 per cent, and monocytes, 0.5 per cent

Although erythroid elements were present, the bone marrow appeared to be almost completely composed of neutrophilic elements (figure 3). Differential counts (table 2, No. 14) revealed that neutrophilic elements represented 90.0 per cent of the cells in the bone marrow while the erythroid elements showed a relative decrease to only 3.6 per cent of marrow cells (normal dogs 43.7 per cent neutrophilic elements, 40.4 per cent erythroid elements)

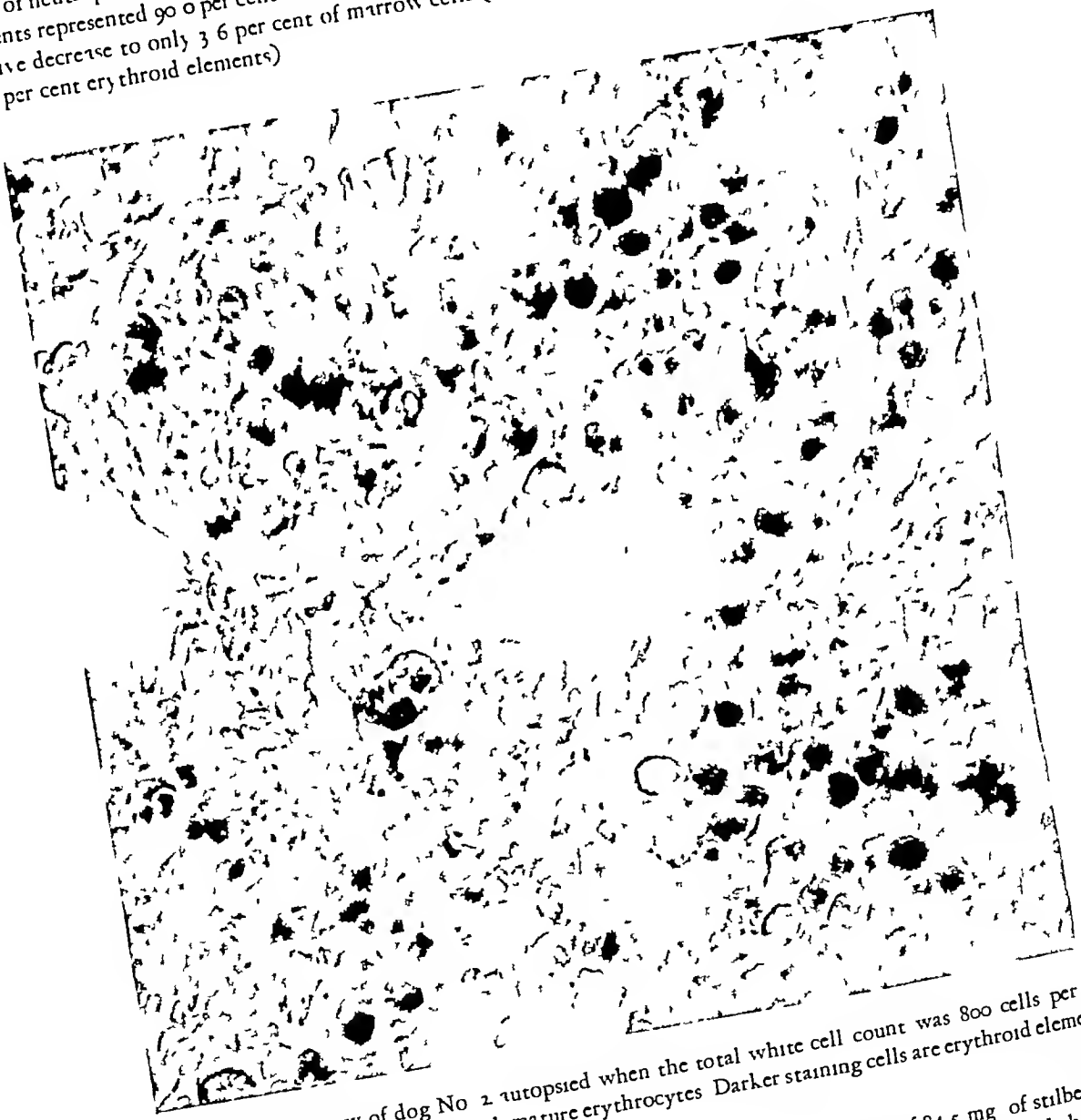


FIG. 4 Bone marrow of dog No. 2 autopsied when the total white cell count was 800 cells per cu mm. Note the extreme congestion with mature erythrocytes. Darker staining cells are erythroid elements.

*Dog No. 9* This animal was treated for twenty-seven days with a total dose of 84.5 mg of stilbestrol. The leucocyte count increased from 11.7 thousand cells per cu mm to 36.8 thousand cells, and then decreased to 21.1 thousand, at which time it was sacrificed. At autopsy, the erythrocyte count was 5.88 million cells per cu mm, hemoglobin, 14.5 Gm per 100 cc, neutrophils, 89.0 per cent, eosinophils, 0.5 per cent, basophils, 0.5 per cent, lymphocytes, 9.0 per cent, and monocytes, 1.0 per cent. The bone marrow of this animal was slightly congested and the majority of cells were neutrophilic elements. Neutrophils were not as predominant as found in the previous dog which was autopsied while the leucocyte count was 46.0 thousand cells per cu mm.

Differential counts (table 2, No. 9) revealed that 86.4 per cent of marrow cells were neutrophilic ele-

ments while only 7.6 per cent of cells were erythroid in nature. These figures should be compared with 43.7 per cent neutrophilic and 40.4 per cent erythroid elements found in the normal dog.

*Dog No. 11* This animal was injected for twenty-five days with a total of 89.0 mg of stilbestrol. The leucocyte count increased from 11.6 thousand cells per cu mm to 34.5 thousand cells, and then decreased to 18.8 thousand cells at the time it was sacrificed. At autopsy the erythrocyte count was 5.88 million cells per cu mm, hemoglobin, 14.0 Gm, neutrophils, 67.0 per cent, eosinophils, 2.0 per cent, basophils, 0.5 per cent, lymphocytes, 30.0 per cent, and monocytes, 0.5 per cent. The majority of the neutrophils were of the older variety—a shift to the right.

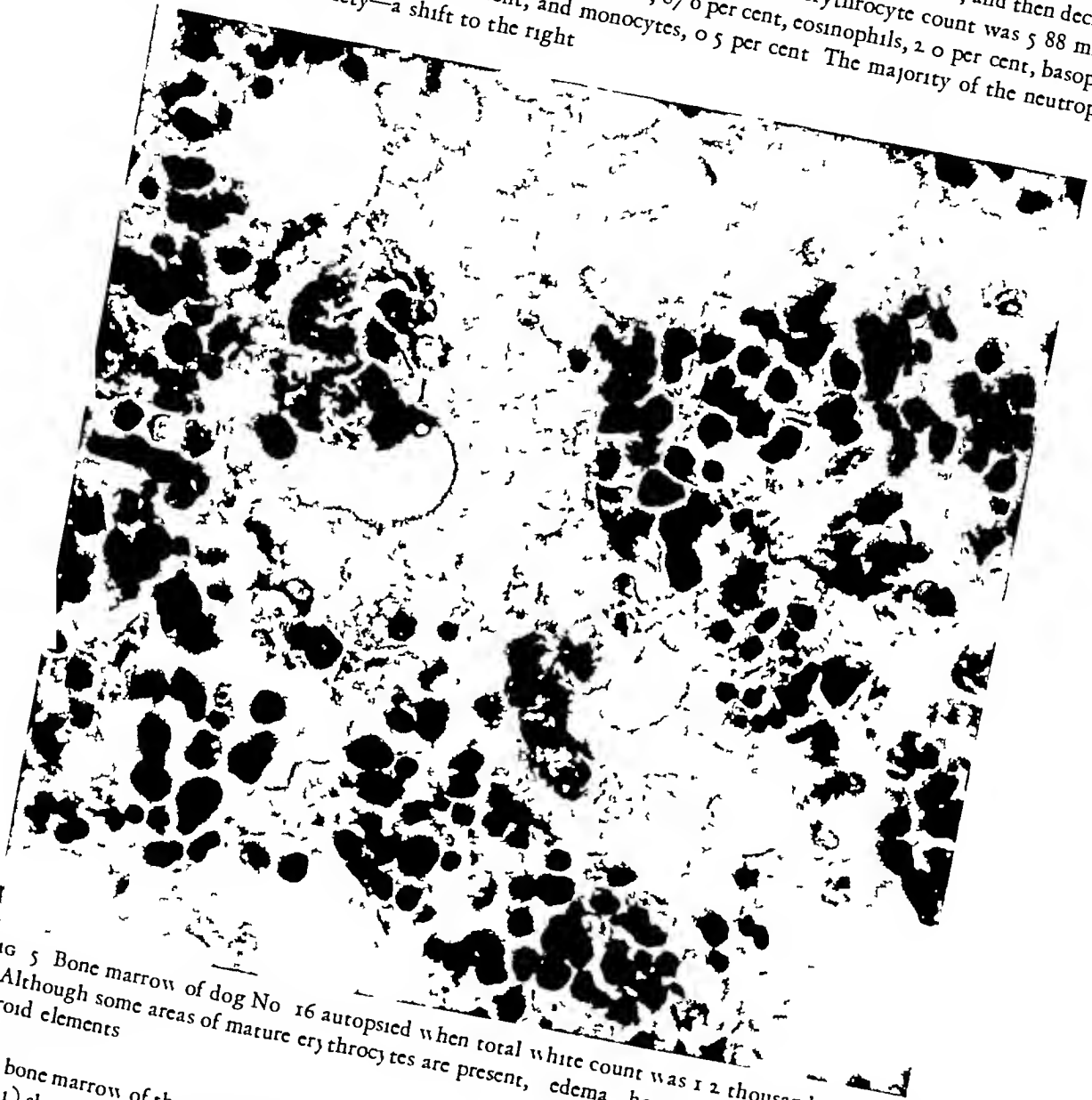


Fig. 5 Bone marrow of dog No. 16 autopsied when total white count was 12 thousand cells per cu mm. Although some areas of mature erythrocytes are present, edema has replaced everything but the erythroid elements.

The bone marrow of this animal looked like marrow from a normal animal. Differential counts (table 2, No. 11) showed, however, that 64.8 per cent of marrow cells were neutrophilic elements and 31.4 per cent erythroid elements.

*Dog No. 12* This animal was treated with 5.0 mg of stilbestrol per day for one hundred and twenty-two days. The leucocyte count increased from 19.2 thousand cells per cu mm to 46.9 thousand cells on the eighteenth day of treatment, decreased to 4.0 thousand cells on the forty-third day of treatment, and then gradually decreased to 0.8 thousand cells on the one hundred and twenty-second day, the day the animal was sacrificed. The erythrocyte count decreased from 7.0 million cells per cu mm to 2.6 million cells, hemoglobin, from

15.2 Gm per 100 cc to 7.0 Gm. The blood smear, made on the last day of treatment, did not contain enough cells to make a complete differential blood count. Of the cells counted (60) 11.0 per cent were neutrophils, 2.0 per cent, eosinophils, and 87.0 per cent were lymphocytes.

The bone marrow of this animal was extremely congested. Most of the area in the bone marrow was taken up by mature erythrocytes (figure 4).

Differential counts (table 2, No. 2) revealed a complete lack of neutrophilic elements. 96.5 per cent of the marrow cells were erythroid elements. There was a considerable deposit of hemosiderin pigment in this bone marrow.

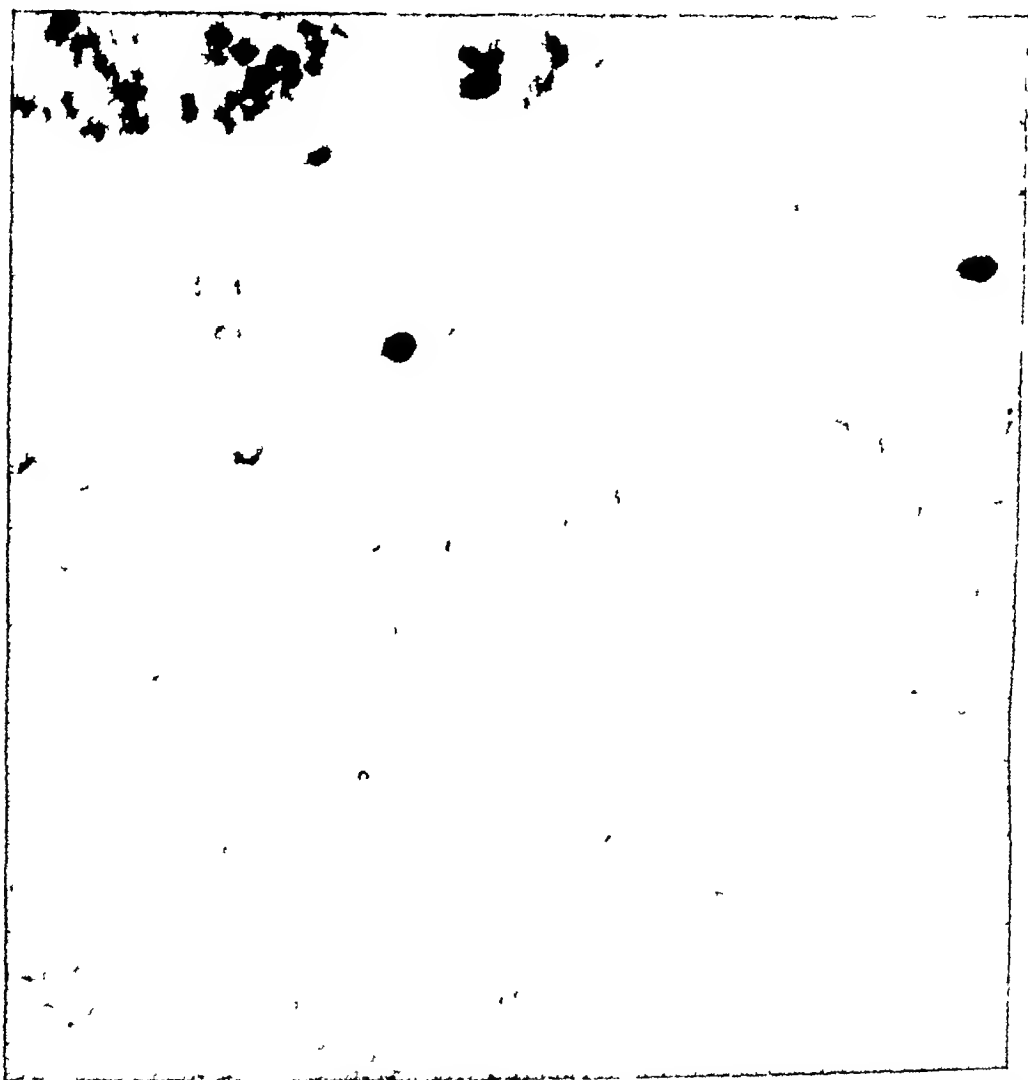


FIG. 6 Bone marrow of dog No. 1 autopsied when the total white cell count was 200 cells per cu. mm. Note the almost complete replacement of the remaining erythroid elements with edema.

*Dog No. 16* This dog was treated for thirty-three days with a total dose of 93.5 mg. of stilbestrol. The leucocyte count increased from 9.5 thousand cells per cu. mm. to 22.7 thousand cells on the twentieth day of treatment. The animal was sacrificed on the thirty-third day when the leucocyte count was 1.2 thousand cells per cu. mm. This animal did not exhibit an anemia. The erythrocyte count was 5.37 million cells per cu. mm. and the hemoglobin was 13.5 Gm. per 100 cc. There were not enough white cells on the blood smears to make a differential count.

The bone marrow of this animal (figure 5) showed the same lack of neutrophilic elements as shown in the previous animal, but the areas of extreme congestion were supplanted by a moderate congestion plus large areas of edema. Erythroid elements stood out more clearly. Active erythropoiesis was present and the bone marrow had considerable hemosiderin deposit.

Differential counts (table 2, No. 16) showed results similar to those found in the previous animal: 97.4 per cent of the marrow cells were erythroid elements while only 0.6 per cent were neutrophilic elements.

*Dog No. 1.* This dog received 5.0 mg. of stilbestrol per day for thirty-four days. The leucocyte count increased from 13.8 thousand cells per cu. mm. to 54.0 thousand cells on the twenty-third day of treatment. The animal was sacrificed when the leucocyte count was 0.2 thousand cells per cu. mm. The erythrocyte count decreased from 6.18 million cells per cu. mm. to 4.40 million. There were not enough cells in the blood smear to make a differential count.

The bone marrow of this animal (figure 6) was almost entirely devoid of cells. The whole marrow was replaced by the "edema" present. There were not enough cells to make a differential count.

In summary, large doses of estrogen are toxic to the bone marrow of adult dogs. Under the influence of estrogen treatment there is a great stimulus to the production of neutrophilic elements (figure 3), with a concomitant increase in the white cells of the peripheral blood. This accounts for the increase in leucocyte count which invariably follows treatment of dogs with large doses of estrogen. At this stage the majority of neutrophils are of the young variety—a shift to the left. Continued estrogen treatment results in a marked congestion of the bone marrow (figure 4), which is accompanied by destruction of white cell elements. This accounts for the marked decrease in the leucocyte count in the blood stream. The neutrophils in the blood are old—a shift to the right. The areas of the bone marrow formerly occupied by mature erythrocytes and the white cell elements are then replaced by "edema" which, for a time, spares erythroid elements (figure 5). Continued treatment is toxic to the remaining erythroid elements so that the marrow becomes almost completely devoid of cellular elements (figure 6).

### DISCUSSION

Estrogens, in large doses, appear to be very toxic to the bone marrow of dogs. Arnold and co-workers<sup>2</sup> have reported results essentially similar while Dougherty, Williams, and Gardner<sup>19</sup> reported, in abstract form, that estrogens produced an aplastic bone marrow.

There are indications that the effects of estrogens on dogs are due to their toxicity to this animal rather than to any physiological estrogenic activity. One adult female dog was treated with daily doses of 5.0 mg. of alpha estradiol and another, of similar size and weight, was treated with 5.0 mg. of beta estradiol, known to have one fortieth of the estrogenic potency of alpha estradiol. Both animals responded to the injections with a great increase in the total leucocyte count followed by a marked decrease.<sup>11</sup> The neutrophils were responsible for these changes. In addition, 2 dogs were treated with daily doses of 0.15 mg. of alpha estradiol,\* an amount which would approximately represent the physiological activity of daily doses of 5.0 mg. of beta estradiol. Except for an initial slight rise in the leucocyte count, these animals showed no ill effects after a year of injections.

It is of interest that injections of 10 mg. of estrogen per day produced no such reaction in monkeys,<sup>11</sup> and there are no indications that the human responds in any such manner.

\* This alpha estradiol was very generously furnished by Dr. Erwin Schwenk of the Schering Corporation.

Dogs respond to estrogens according to their physical condition. While one animal will live for one hundred twenty-six days receiving a daily dose of 50 mg of estrogen and exhibit several increases and declines of the leucocyte count, other dogs will die in thirty days with the same dose. One dog, not reported, received only 0.5 mg of alpha estradiol per day and died on the seventy-sixth day of treatment with a bone marrow completely devoid of cellular elements. This bone marrow resembled that shown in figure 6. At autopsy, this animal was found to be suffering from a respiratory infection and had many intestinal parasites.

Estrogens are toxic to the bone marrow of adult dogs. The toxic dose level differs from animal to animal depending upon its physical condition.

### SUMMARY

Large doses of estrogens have a profound effect on the bone marrow of adult dogs. The initial reaction is a great increase in the number of neutrophilic elements in the bone marrow. These neutrophils are released into the blood stream causing a marked rise in the total white cell count. This is followed by congestion of the bone marrow and a destruction of the white cell elements in these marrows. Congested areas and locations formerly occupied by white cell elements are replaced by edema, leaving erythroid elements intact. This accounts for the marked drop in the total white cell count in the blood stream. This is followed by destruction of remaining erythroid elements in the bone marrow and replacement by "edema" until a stage is reached where practically no cells can be found in the marrow.

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# THE FREQUENCY OF MEGAKARYOCYTES IN AUTOPSY SECTIONS

By ROBERT BRILL, M D ,\* AND M M HALPERN, M D †

THE PRESENCE of megakaryocytes in tissue sections has been frequently reported as a significant finding<sup>1-7</sup> According to Petri<sup>8</sup> who reviewed the literature, Muller was the first to note the occurrence of megakaryocytes in organs Aschoff often observed them in the lungs in infectious diseases, and less frequently in the kidneys, liver and myocardium Bunting,<sup>3</sup> in a study of bone marrow and lung sections in 11 cases of Hodgkin's disease, found megakaryocyte nuclei in the lung capillaries of all cases, in 4 of these he thought megakaryocytes were more numerous than to be expected in cases with marked leukocytosis There was an increase in the number of marrow megakaryocytes in 3 of the 5 cases studied

In cases of active tuberculosis, Medlar and Sasano<sup>6</sup> noted numerous megakaryocytes in the lungs Orbison<sup>1</sup> reported a case of lobar pneumonia with widespread distribution of megakaryocytes in the lungs and many other organs Goroncy<sup>7</sup> found megakaryocytes in the lesser circulation (lungs) in 61 per cent of his series of autopsies, in 13 per cent they were observed also in one or more of the organs supplied by the greater circulation, in only 3 of these necropsies were they found in the lungs, spleen, liver and kidneys of the same case Petri<sup>8</sup> also lists their presence in the bone marrow capillaries and sinusoids of the lymphoid tissue Downey and Nordland,<sup>5</sup> in a case of myeloid leukemia, observed megakaryocytes in the cortex of the adrenals and the surrounding fatty tissue, as well as in other organs

Most investigators<sup>2, 9-15</sup> feel that megakaryocytes appear in the organs in conditions associated with increased hematopoietic activity, a viewpoint first expressed by Aschoff Hewer,<sup>10</sup> in a case of osteosclerotic anemia, observed many megakaryocytes in the spleen Because of their absence in the lung sections, he concluded that in this case they were formed in the spleen Downey, Palmer and Powell<sup>4</sup> found megakaryocytes in liver and spleen biopsies in a case of atypical myelosis, and thought they were produced in situ Klemperer,<sup>17</sup> in his report of cavernomatous transformation of the portal vein, noted numerous megakaryocytes in the spleen He was undecided as to whether or not they were "carried to the spleen by the blood stream, because even when present in great numbers in the venous sinuses they were not found in the arteries" Howell and Donahue<sup>18</sup> maintained that megakaryocytes developed in the lungs from "myeloblastic cells in the same manner as they do in the liver, spleen and bone marrow" However, Jordon<sup>12</sup> did not agree with this conclusion

There have been several reports of the unusual finding of intact or fragmented megakaryocytes in the smears of peripheral blood<sup>5, 13, 15, 19</sup> Minot<sup>19</sup> stated that they were present in the peripheral blood smears in cases of myelogenous leukemia, polycythemia, and rarely in "simple leukocytosis," lobar pneumonia, Hodgkin's

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disease and sepsis. He believed that it was indicative of "intense bone marrow strain." Downey<sup>5</sup> stated that megakaryocytes appeared after splenectomy in the peripheral blood smears from a case of myelogenous leukemia.

This problem was pursued at the suggestion of Dr. William Antopol and Dr. Lester Goldman, who had observed the frequency of megakaryocytes in paraffin sections and felt that they were partially filtered out in the lungs and that the spleen had a special affinity for actively arresting and fixing them therein. It was decided to study megakaryocyte occurrence in the tissues of 50 autopsy cases.

#### METHODS AND MATERIAL

The first 27 cases chosen for study were cases such as bacterial endocarditis, pneumonia or metastatic carcinoma, in which megakaryocytes were expected to be found. Then 7 autopsies were selected with no history of infection, neoplasm or blood dyscrasia, in which tissue megakaryocytes were not expected to be found. To round out the number an additional 16 random cases were studied.

The diagnoses in the 50 cases were as follows: 9 cases of neoplasm with metastases including bone, 7 of pneumonia, 7 of rheumatic heart disease, 3 of acute bacterial endocarditis, 3 of subacute bacterial endocarditis, 3 of pulmonary tuberculosis, 3 of cardiovascular-renal disease, 2 of Hodgkin's disease, 2 of leukemia, and one each of periarteritis nodosa, Libman-Sacks disease, chronic glomerulo-nephritis, granulomatous ulcer of the buttock, hypoglycemic shock, methyl alcohol poisoning, syphilis of the central nervous system, Landry's paralysis, traumatic death, primary amyloidosis, and a postoperative death.

Studied, but not included in this group of 50, were an additional 4 cases which showed extramedullary hematopoiesis.

With routine hematoxylin and eosin stain, the appearance of the megakaryocyte in the peripheral capillaries differs somewhat from that in the bone marrow. In the latter site the megakaryocyte is round, it possesses an abundant, homogeneous pink cytoplasm, the edge of which may or may not be irregular, the nucleus is large, vesicular or pyknotic, and may have few or many lobules. In the tissue capillaries, the megakaryocytes assume various shapes, being distorted by the channels in which they lie, their cytoplasm is often not discernible and the nucleus may appear naked (fig. 1). To a great extent, identification of megakaryocytes depends on their intravascular location and their enormous size, and especially on the large multilobulated nucleus which is most often pyknotic. Megakaryocytes with abundant cytoplasm are occasionally present, particularly in the wider channels (fig. 2).

In the lungs, the megakaryocytes are most frequently found in the capillaries of the alveolar septa. The glomerular tufts are their usual site in the kidneys, however, they are also seen in the interstitial capillaries. In the liver and spleen sections they are usually found in the blood sinuses. In heart sections they appear in the capillaries between the muscle fibers.

When megakaryocytes were found in the organs, a rough estimate of their number was recorded as being one to three plus. When only two or three megakaryocytes were found in the examination of a whole paraffin section, about 1 cm



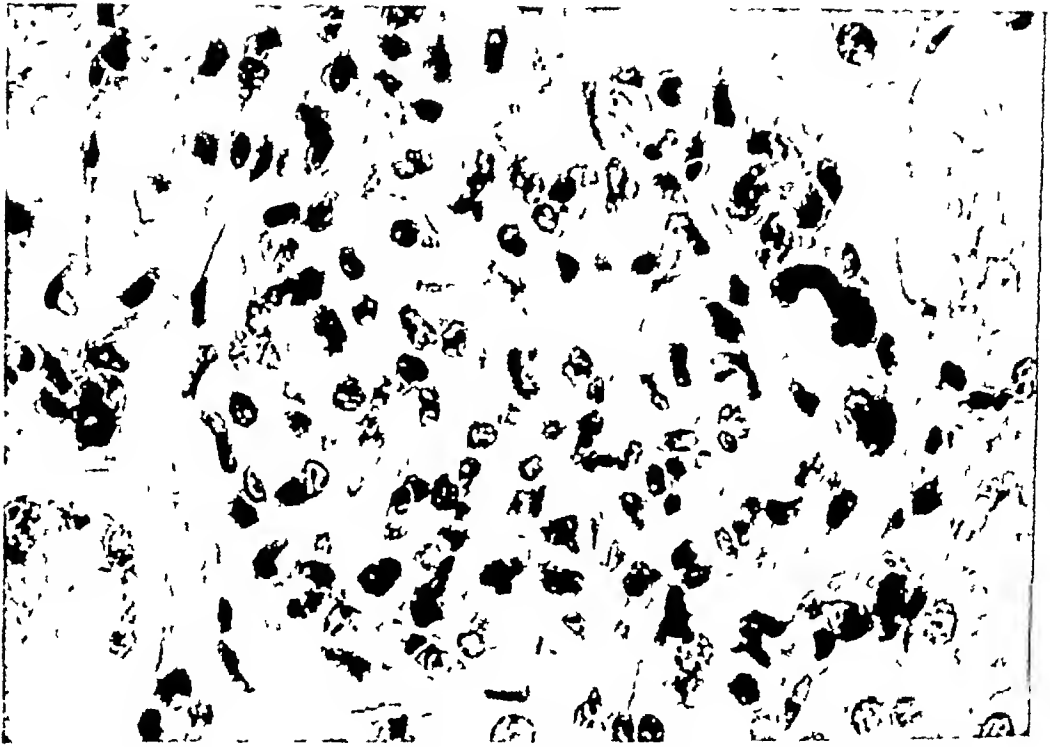


FIG. 1 ALMOST NAKED MEGAKARYOCYTE NUCLEUS IN A GLOMERULAR CAPILLARY

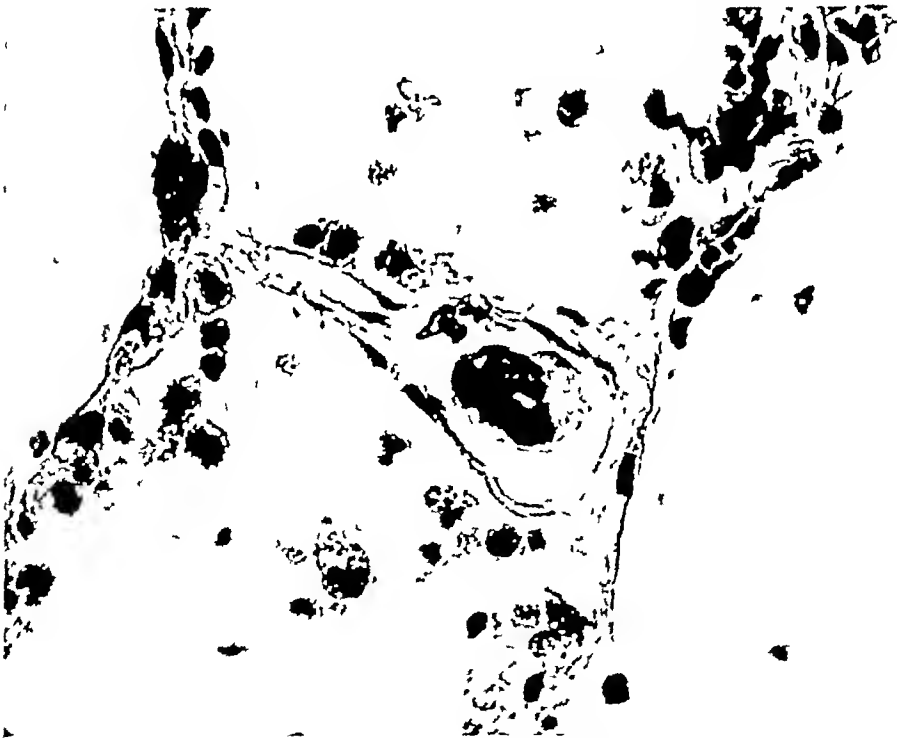


FIG. 2 MEGAKARYOCYTE WITH ABUNDANT CYTOPLASM IN THE PULMONARY CIRCULATION

square, a one plus was recorded. Those cases with large numbers of megakaryocytes, averaging 1 or more per low power field (16 mm objective and 10 $\times$  ocular), were considered three plus. Two plus represented the intermediate group. No attempt

was made to use absolute numbers first, because of the difference in anatomical blood supply in the different organs, secondly, because of the varying pathologic changes in the same organ in different cases

### RESULTS

In all of the 50 cases studied, megakaryocytes were demonstrated in the lungs. Their presence in smaller percentages was noted in the spleen, kidney, liver and heart (table 1). Megakaryocytes were noted in the adrenals in two cases, and in the pancreas and stomach in one instance each, but these are not included in the table.

TABLE 1 — *Incidence of Megakaryocytes in Various Organs*

Organs examined	No. of cases examined	No. with meg's present	Percentage incidence
Lungs	50	50	100
Spleen	45	28	62
Kidney	47	17	36
Liver	47	16	34
Heart	45	6	13

TABLE 2 — *Simultaneous Incidence of Megakaryocytes in the Organs in Forty Cases*

Lungs	Spleen	Kidney	Liver	Heart	No. of cases
P	O	O	O	O	16
P	P	O	O	O	6
P	P	P	O	O	3
P	P	P	P	O	7
P	P	P	P	P	5
P	P	O	P	O	2
P	P	O	O	P	1

P megakaryocytes present

O megakaryocytes not found

Sections of all the five organs mentioned in table 1 were available for study in only 40 of the 50 cases. The simultaneous occurrence of megakaryocytes in one or more of these five organs of each of these 40 cases is listed in table 2. It will be noted that megakaryocytes were found in the lungs alone in 16 cases, and in the lungs and spleen in 6. There were 12 cases with megakaryocytes present in the lungs, spleen, kidney and liver, and in 5 of these, megakaryocytes were also seen in the heart. Smaller numbers of other combinations are listed in table 2.

There appeared to be some correlation between the concentration of megakaryocytes in the lung capillaries and their occurrence in other organs (fig. 3). Seventeen cases demonstrated a one plus megakaryocyte concentration in the lungs; no megakaryocytes were found in the other organs in 11 of these cases, in the other 6 cases, they were found only in the spleen or in the spleen and one other organ.

Eleven cases showed a two plus megakaryocyte concentration in the lungs.

Five of these had no megakaryocytes in the other organs. In 4 more, they were demonstrated only in the spleen or the spleen and one more organ. In the 2 cases with involvement of all five organs, the megakaryocytes were numerous in the lungs, but not sufficiently numerous to fall into the three plus category.

All 12 cases with a three plus concentration in the lungs had megakaryocytes also in other organs. In 3 cases all five organs were involved, and in 7 more, megakaryocytes were demonstrable in all but the heart.

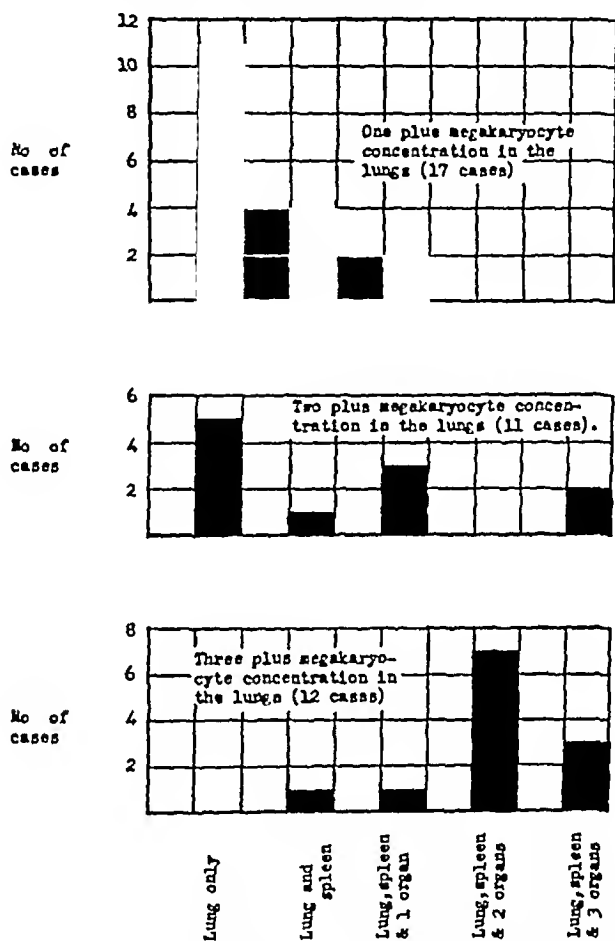


FIG 3 CORRELATION BETWEEN THE MEGAKARYOCYTE CONCENTRATION IN THE LUNGS AND THE NUMBER OF OTHER ORGANS INVOLVED

One interesting finding was the constant presence of megakaryocytes in the spleen, whenever there was involvement of organs besides the lungs (table 2).

The diagnoses in the 12 cases (table 2) with involvement of four or five organs were pneumonia, acute or subacute bacterial endocarditis and carcinoma with metastases.

In the 4 cases with extramedullary hematopoiesis, it was possible to show, in one or another of the organs, a megakaryocyte concentration roughly as great as, or greater than that in the lungs.

#### DISCUSSION

The greatest incidence of extramedullary megakaryocytes appears to be in the lungs. This fact suggests, in agreement with earlier investigators, that their

presence is dependent upon their delivery into the peripheral blood from the bone marrow and their subsequent filtration by the pulmonary capillary bed

From the data presented one might conclude that with more megakaryocytes entering the blood stream from the marrow, more will reach the lungs, and in turn, the more that filter through the pulmonary capillary bed, the more will be carried to other organs

One might also conclude that under normal conditions variable numbers of megakaryocytes enter the blood stream from the bone marrow and can be demonstrated in the lung capillaries. The number of these is frequently increased in disease processes. Those megakaryocytes passing the pulmonary capillary bed are most frequently demonstrated in the spleen

#### SUMMARY

- 1 Megakaryocytes can be demonstrated with great frequency in the viscera
- 2 Megakaryocytes were present in the lungs in all of 50 autopsies studied
- 3 They were next most frequently found in the spleen
- 4 The simultaneous occurrence in the various organs, in the absence of extramedullary hematopoiesis, is roughly related to the concentration in the lungs
- 5 Under normal conditions megakaryocytes in small numbers circulate in the blood stream

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# FEMORAL BONE MARROW BIOPSY IN THE ALBINO RAT

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IN THE course of another investigation we became interested in studying the myelogram of the rat with a view to observing changes which might occur as the result of experimentally induced anemias. The scanty published work on this subject has been recently referred to by Vogel (1947). She has also developed a method for obtaining marrow and has given a detailed description and classification of the cell types. The limitation of her method is that the animal must in each instance be sacrificed. The present investigation was therefore directed towards the development of a reliable method by which repeated marrow studies of one animal could be made.

## METHOD

Marrow films were made from twelve healthy albino rats of the Wistar strain, aged 4 to 6 months. The rats were fed on a diet which we have found to maintain them in good condition, and to permit normal growth and reproduction (table 1).

To obtain marrow samples, the skin over the femur is depilated by clipping short and then swabbing with 40 per cent sodium sulphide. The animal is anesthetized with ether and tied on its side. Aseptic technic is used and a 2 cm incision made along the axis of the femur. The muscles are then separated to expose the widened upper end of the femur, which can be grasped with a pair of toothed dissecting forceps. Using a sharp 20-gage needle as an awl, a hole is drilled into the marrow cavity. A cannula, previously prepared by filing the bevel and point from a 20-gage needle, and mounting it on a 2 ml all glass syringe, is then introduced into the marrow. Before introduction, human plasma is aspirated into the syringe and then expelled, so that plasma remains in the cannula but not in the syringe. Suction is applied until marrow just shows in the syringe and the cannula is then withdrawn. The marrow, already mixed with plasma, is expelled onto a series of slides, and films are made and quickly air-dried in the usual manner. From six to eight films can be made without difficulty. The muscles are brought together with a single cotton suture. The skin is closed, with either a continuous eversion cotton suture or Michel clips. The wound is sealed with collodion. No other dressing is applied. Clips are removed in forty-eight hours but sutures may be left. The animals have no disability after the operation and the wound is healed within a week.

## RESULTS

A second biopsy can be made from the same femur after a fortnight. Marrow films from the second femur can be made as soon as two days after biopsy of the first femur. The range of cellular distribution is not altered in these instances. By using the femurs alternately, satisfactory examinations of the marrow can be made at weekly intervals.

From the Nuffield Department of Medicine, Radcliffe Infirmary, Oxford, England

TABLE 1 — *Diet*

crude casein	25
corn starch	68
cod liver oil	2
arachis oil	2
mineral salts	3
	<hr/> 100

## Supplements

thiamine	100 gamma daily
riboflavine	200 gamma daily
nicotinic acid	1 mg daily
pyridoxine	100 gamma daily
pantothenic acid	200 gamma daily
2-Methyl 4-naphthaquinone	40 gamma daily
choline	10 mg daily
-tocopherol	3 mg weekly
vitamin A 2000 units	twice weekly
vitamin D 200 units	twice weekly

TABLE 2 — *Femoral Bone Marrow of the Adult Albino Rat*    *Distribution of the Cellular Elements*  
Leuko-Erythro Ratio = 1.8

Cell Type	Distribution %	
	Range	Average
Blast cells	4- 1 8	1 0
Promyelocytes	8- 3 4	1 8
Neutrophilic myelocytes	2 2- 5 0	3 5
Neutrophilic young forms	5 6- 9 2	7 6
Neutrophilic staff cells	8 2-14 0	11 2
Neutrophilic mature forms	16 0-28 6	21 4
Eosinophilic myelocytes	4- 2 4	1 6
Eosinophilic mature forms	2 6- 7 0	4 2
Basophiles	0- 4	1
Lymphocytes	6 6-24	15 1
Monocytes	1 0- 2 2	1 6
Plasma cells	4- 1 0	9
Proerythroblasts	4- 1 8	1 4
Basophilic erythroblasts	3 2-10 0	6 2
Polychromatic erythroblasts	10 8-27 2	21 1
Orthochromatic erythroblasts	0- 4	1
Megakaryocytes	0- 8	3
Macrophages	0- 4	2
Mast cells (Kugelhaufen)	0- 2	1
Cells in mitosis	2- 8	6

Satisfactory films were always obtained by this method despite the fact that human plasma will agglutinate rat erythrocytes \* Better films were made by mixing

\* Human plasma was used in preference to rat plasma because it was readily available and gives satisfactory results (Endicott, 1945)

marrow with plasma in the cannula than by using undiluted marrow, or by diluting the marrow on the slide in a drop of saline or plasma

Differential counts were made from films prepared by this method, 1000 cells being counted in each instance May-Grunwald-Giemsa staining was used throughout Although there was considerable variation in distribution, the morphology did not vary in the series The counts are summarized in table 2, employing a modification of the classification used in this department for reporting on sternal marrow smears This classification differs in a few respects from those used by other authors

Primitive cells, which cannot be differentiated with certainty, we have labelled "blast" cells, the majority are presumably myeloblasts

Neutrophilic granulocytes of greater maturity than myelocytes have been classified as young forms, staff cells and mature neutrophils We agree with Vogel (1947) that the ring cell is more mature than the myelocyte, but we find that the nucleus of the granulocyte, at all stages of maturity beyond the myelocyte, may have a ring formation It is therefore necessary to consider all the characteristics of a ring cell before assessing its maturity Most of the ring cells are here classified as young forms, together with Vogel's metamyelocytes

Eosinophilic granulocytes have been classified into two groups only myelocytes and mature forms Ring forms are also present in the eosinophil series

No precursors of basophilic granulocytes were observed

Cells of the erythropoietic series have been divided into four groups with descriptive names The differentiation of the proerythroblast from other "blast" cells is difficult and cannot be accomplished with certainty by the present methods

There was no sex difference in the myelograms

#### SUMMARY

A technic for repeated femoral bone marrow biopsy in the rat is detailed

Differential counts confirmed the distribution of the cellular elements as described by other authors

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# THE OCCURRENCE AND SIGNIFICANCE OF "MOTILE" ERYTHROCYTES IN HUMAN BLOOD AND MARROW IN ANEMIC STATES\*

By PAUL H. RALPH, M.D.†

IT HAS frequently been observed that fixed films of blood from macrocytic anemias, e.g., pernicious anemia, are characterized by poikilocytosis to a degree not seen in other anemic states. The study of living blood presents new facts of interest to the cytopathologist which contribute to the explanation of this phenomenon.

The dark-field microscope is particularly suited to the study of living blood cells since the smallest organoids are visible without stains or other alterative treatment and the most delicate of membranes appear as lines of varying thickness and refractivity.<sup>6</sup> Consequently, all descriptions in this paper, unless otherwise specified, are of the dark-field appearance of the structures concerned. Because of better reproducibility, the photographs are given as negative rather than positive prints. Artifact formation was kept at a minimum by using only freshly drawn material and by handling it with the greatest care.

In a number of anemias of varying etiology (Hodgkin's disease, leukosarcoma, pernicious anemia, et al.) it was noted that some erythrocytes of the peripheral blood exhibited irregular cell surfaces. The membrane of these cells (figs 1-5, plate I) was less thick and refractive than that of the red blood cells of typical discoid shape. This appearance of the cell surface denotes a lower concentration of hemoglobin and a more primitive condition of the cytoplasm.<sup>6</sup> Although most of these cells contained mitochondria and neutral red-staining granules‡ not all of the cells containing such organelles exhibited motility. Few if any erythrocytes from normal blood contain such structures.<sup>6</sup>

It was noted that this irregular form of the cytoplasm was constantly changing. In the least primitive of these cells the alterations took the form of small shallow notches that repeatedly appeared in their surface at from one to four places and slowly disappeared. In the more primitive forms (figs 1-5, plate I) these constrictions were much deeper and divided the cell into lobes or pseudopods which were slowly protruded and retracted. Coincident with these surface changes the organoids of the cell, sometimes tangled into a knot, slowly moved about as though carried by currents within the cytoplasm. Occasionally in the most primitive erythrocytes a tiny granule-free area was present, surrounded by a group of neutral red-staining granules about which the mitochondria were loosely aggregated—the centrosphere. The sum of all these activities constitutes something that simulates

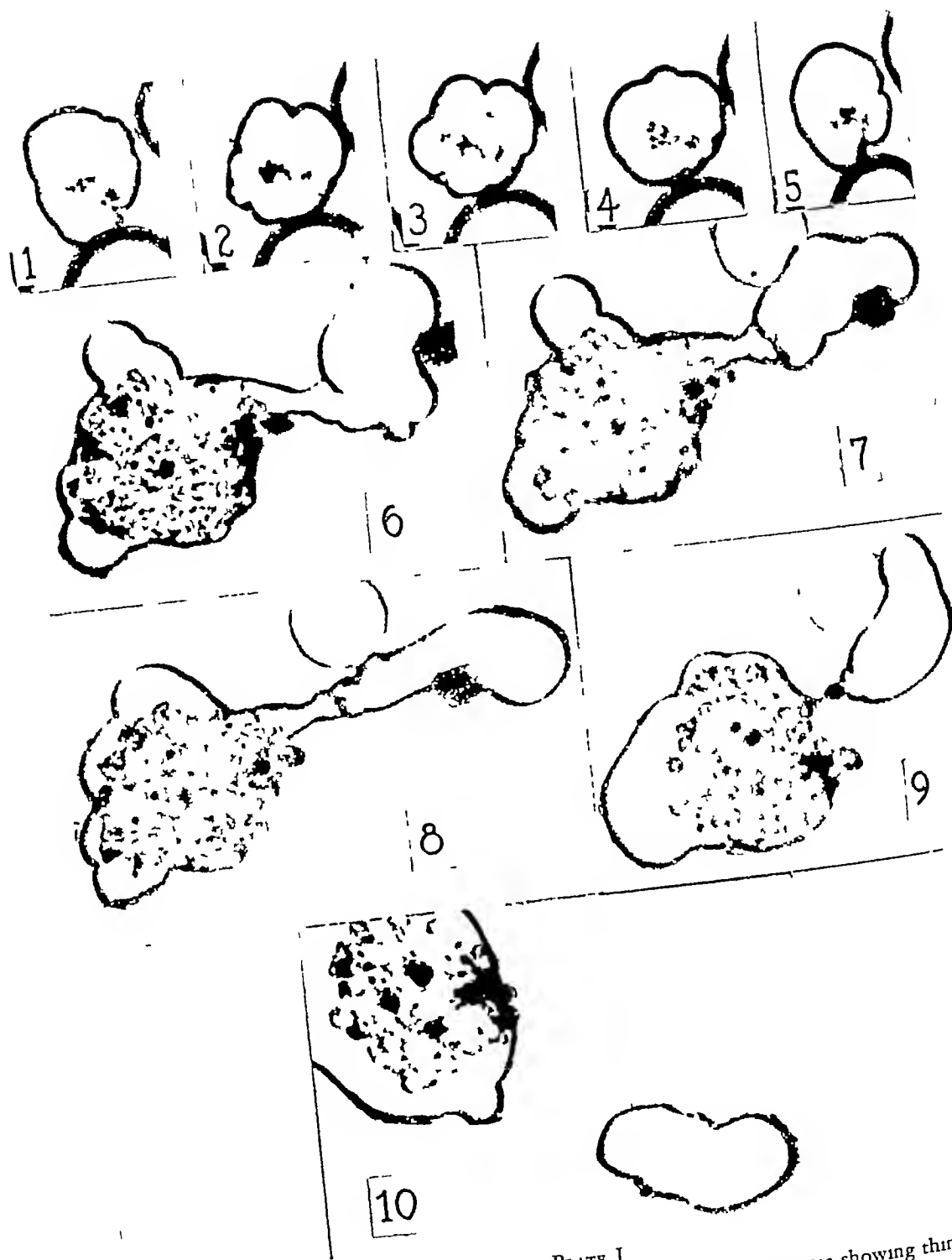
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\* This work was supported by a fellowship from the Horace Rackham School of Graduate Studies of the University of Michigan and done under the direction of Dr. C. A. Doan of Ohio State University Medical School.

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‡ Not to be confused with the vacuole or granules of reticulum induced by high concentrations of stain.<sup>6</sup>





# PLATE I

FIG 1 Motile primitive erythrocyte from a patient with pernicious anemia showing thin, irregular outline, rod-like mitochondria and neutral red-stainable granules 1500 X

FIG 2 Same cell as in fig 1, one minute later

FIG 3 Same cell as in fig 1, two minutes later

FIG 4 Same cell as in fig 1, four minutes later

FIG 5 Same cell as in fig 1, six minutes later

FIG 6 Megakaryoblast from bone marrow of patient with Hodgkin's syndrome showing the formation of a large pseudopod with a partial constriction of the cell membrane at its base 1500 X

FIG 7 Same cell as in fig 6, three minutes later Note completion of cytoplasmic constriction

FIG 8 Same cell as in fig 6, six minutes later Note apparent effort of the pseudopod to detach itself from the parent cell

FIG 9 Same cell as in fig 6, thirty-six minutes later Bud is attached to parent cell by thin, slightly refractive thread

FIG 10 Same cell as in fig 6, eighty-seven minutes later Bud has become detached and continues to exhibit motility

amoeboid movement. It differs from amoeboid movement in that the bud does not move from place to place and that the motility is not related to any vital function such as food getting, phagocytosis of debris, etc.

In order to study the origin of such cells, sternal marrow aspirations were obtained from selected patients whose peripheral blood contained large numbers of 'motile' erythrocytes. All such specimens of marrow contained megaloblasts\* with reduced amounts of hemoglobin in their cytoplasm, displayed the same cell membrane refractivity and types of mitochondria and neutral red-stainable granules that distinguished the motile erythrocyte in the peripheral blood. Some were polynucleate (fig. 14) and contained more than one central apparatus. In some cases multipolar mitoses could be seen, with as many as seven centrospheres clearly marked out by the clustering of neutral red-stainable granules. These cells gave a positive reaction to the benzidine test for hemoglobin.<sup>5</sup> After a short time for adjustment to *in vitro* environment, these cells begin to extrude slowly and to retract one or more large blunt pseudopodia. Gradually the base of such pseudopods might narrow (fig. 6, plate I) and when constriction became sufficiently acute, an actual cleavage of the cytoplasm occurred (fig. 7, 8, plate I, fig. 13, plate II). This cleavage at first involved only the hemoglobin-laden endoplasm of the cell. The thin, weakly refractive ectoplasmic membrane at the surface<sup>6</sup> might remain intact for some time and be visible as a thin gray thread holding the budded erythroplastid to its parent cell (fig. 9, plate I). This strand eventually broke and the newly formed red cell left the mother cell by amoeboid-like movement (fig. 10, plate I). These buds might occur at any place on the cell surface, but there was a tendency for them to be formed near the centrosphere, in which case frequently not only mitochondria but some of the neutral red-stainable granules (fig. 13, plate II), and a part or all of the centrosphere were contained in the bud.

The movement of the newly formed erythroplastid differs from that of the more mature units in the circulation, in that it appears to be more active and the cell moves about from place to place. The motility gradually diminishes in *in vitro* preparations, but it has been seen to persist in individual plastids for as long as five hours after separation from the parent cell.

The formation of buds from leukocytes and connective tissue cells, described as clasmotocytolysis by Ranvier<sup>7</sup> and as pictured by Cunningham, Sabin, Doan<sup>1</sup>, may be of several types and due to a variety of causes. Although a detailed description of these changes is the subject of a further communication, they are here briefly characterized for the sake of comparison.

When exposed to *in vitro* conditions for considerable lengths of time, dark-field illumination demonstrates that the cytoplasmic membrane of living cells becomes much more refractive, thicker and less labile. Eventually the cell becomes permeable to its milieu and imbibes fluid. The cytoplasm becomes markedly less viscous, as evidenced by the acceleration of Brownian movement of organoids within it, and many tiny new granules may be precipitated in the otherwise homogenous

\* The term megaloblast is here intended to include the megaloblast as described by Jones,<sup>3</sup> et al. and the morphologically similar giant erythroid elements found in a variety of dyscrasias. They both exhibit the motility and budding described in this paper.

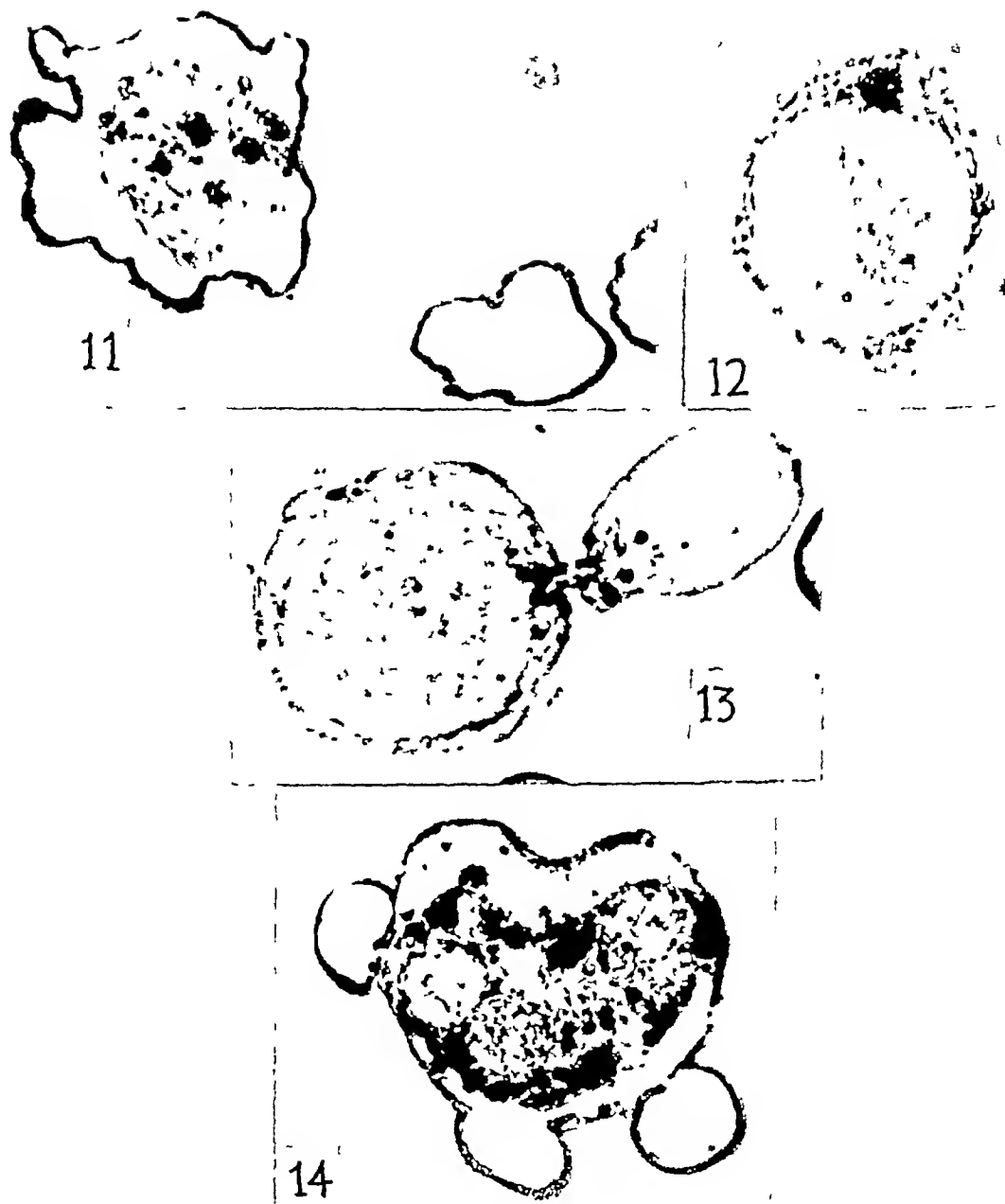


PLATE II

FIG 11 Same cell as in fig 6, one hundred and seventy minutes later Motility continues

FIG 12 Brightfield photograph of megaloblast from patient with Hodgkin s syndrome supravivally stained with janus green B and neutral red Note mitochondria surrounding nucleus and cluster of neutral red-staining granules about central apparatus at upper edge of nucleus 1500 X

FIG 13 Brightfield photograph of bud forming from megaloblast in pernicious anemia Note that base of bud is opposite the central apparatus and that the bud contains rod-shaped mitochondria and neutral red stainable granules 1500 X

FIG 14 Tri-nucleate megaloblast from patient with Hodgkin s disease forming three erythrocytes by budding 1180 X

cytoplasm Amoeboid movement may persist in cells in which these changes have taken place, although it is sluggish and altered qualitatively Portions of the cell

membrane frequently round up and form vesicles which bud from the parent cell. They contain fluid of very low viscosity, organoids and precipitates, and are not capable of amoeboid movement. Clasmatoocytes are characterized by the irreversible extrusion of long tubular, worm-like pseudopodia which may or may not separate from the parent cell. This lytic type of budding is due to alterations in permeability of the cell membrane, and is a part of the death process. The budding of the megakaryoblast differs from that just described, in that it takes place in the living body, the bud is motile, carries out its normal function, i.e., transport of oxygen, and the parent cell does not exhibit moribund changes.

Primitive cells from connective tissue, lymph nodes, spleen and marrow, leukemic and sarcomatous cells and normal, but immature cells mobilized into the peripheral blood by unusual stimuli, are frequently delicate and at the same time turgid and incapable of amoeboid movement. They often fragment without exhibiting many of the lytic changes previously described. The bud produced contains the organoids of the parent cell and exhibits the characteristics of the intact parent cytoplasm. It differs from the budding described for the red cell in that the bud is not motile, serves no useful function and is produced by the mechanical action of an unsuitable environment upon the cell.

The budding of platelets from megakaryocytes, although poorly understood is differentiable from the budding of megalo- and normoblasts because of qualities of the thromboplastid itself. The platelet is an extremely labile thixotropic system which changes irreversibly from a rigid gel to a sol on contact with any wettable surface. Eventually vesicles bud from its surface similar to those formed by the action of *in vitro* conditions upon normal cells.

The question then arises, are these "motile" erythrocytes to be construed as specific entities pathognomonic of the disease in which they are found or are they merely evidence of a greater or lesser "left shift" of erythroid elements in the marrow with a larger percentage of the circulating cytoplasmic units derived from more primitive cells. The mouse (Klieneberger<sup>2</sup>) and the rabbit (Seyfarth<sup>9</sup>) normally have many more circulating reticulocytes than does the adult human. In a study of normal adult rabbits the author found some quite motile erythrocytes in every sample of blood examined. They contained mitochondria and neutral red-staining granules and were in every way similar to those found in human macrocytic anemia except that there was not the great variation in size and shape nor the pronounced hypochromia. The same phenomenon was found in the mouse except that fewer of the cells showed motility. Young animals of both species showed more motile erythrocytes than did the adults.

#### DISCUSSION

The *normal* process by which a mammalian erythrocyte is produced is one of budding in which the parent cell, i.e., the normoblast, becomes separated into two fragments. One contains a pyknotic nucleus, a shred of cytoplasm, and perhaps a few cellular organoids, the other is a disc of hemoglobin-laden cytoplasm with a cell membrane and occasionally a few mitochondria and neutral red-staining granules. This process has been seen to occur *in vitro* by the author. It is, therefore,

perhaps inaccurate to speak of the normoblast as "extruding" its nucleus merely because the division is usually unequal and the nucleus-containing fragment smaller and sometimes apparently devoid of cytoplasm. In the normal human adult the reticulocyte exhibits no motility. This is because the normoblast from which it buds is so mature as to have lost this quality. Under conditions in which the marrow is unable to supply erythrocytes from mature normoblasts, younger and younger cells, with less hemoglobin and with other evidences of immaturity such as the presence of mitochondria and neutral red-staining granules, a more fluid cytoplasm, and amoeboid movement are called upon to supply these small packages of respiratory pigment, the erythrocytes. Naturally, these red cells will to a certain extent exhibit the characteristics of their parent, i.e., hypochromia, motility, content of organoids, etc.

Werner Schultz<sup>6</sup> in a report based on a single case of untreated pernicious anemia described the formation of erythrocytes (blastopodiocytes) by localized budding of the cytoplasm of megaloblasts. He regarded this budding as the true origin of the poikilocyte, and as a "pathological regeneration" limited to the megaloblast of pernicious anemia in the human adult and, consequently, a point of "functional" differentiation between this cell type and the "macroblast" of some other anemic states. We cannot support his conclusions. First, we have found this budding to occur in a variety of conditions other than pernicious anemia. Second, poikilocytosis and "motile" erythrocytes are by no means limited to pernicious anemia but may occur in the human adult in a variety of anemic states and in the rabbit and mouse under normal conditions. The presence of large numbers of poikilocytic and motile erythrocytes is due to the absence of normal blast forms from which they may be budded.

#### SUMMARY

1 With the aid of supravital studies and dark-field illumination, "motile" erythrocytes (capable of self initiated amoeboid-like change in form and, in some cases, movement from place to place) may be found in the peripheral blood of cases of anemia, particularly those of the macrocytic variety.

2 These motile erythrocytes are usually hypochromic, contain mitochondria and neutral red-stainable granules, all of which are attributes of normal but immature red cells.

3 Motile erythrocytes similar to those found in the blood of human patients with anemias are found in the blood of normal rabbits and mice.

4 In the marrow of patients with macrocytic anemias, megaloblasts and megalocytes may be found which give rise to motile erythrocytes by budding.

5 Poikilocytosis, motility, paleness, content of mitochondria and neutral red stainable granules, and origin by budding from a large parent cell do not differentiate the erythrocytes of pernicious anemia from those of other anemic states.

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# CASE REPORT

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## ULCERS OF THE LEG IN MEDITERRANEAN DISEASE

B<sub>3</sub> J EARLE ESTES, M D , EUGENE M FARBER, M D , AND J M STICKNEY, M D

**M**EDITERRANEAN disease, better known as Cooley's anemia, has been recognized as a distinct clinical entity for only a little more than two decades. In spite of this fact, an extensive literature on this comparatively rare disease has been developed and has recently been reviewed by Wolman and Dickstein.<sup>1</sup> It was not recognized until recent years that Mediterranean disease occurred in adult persons as well as in children. What was formerly considered to be a severe, invariably fatal disease of childhood has been found to be a disease which varies greatly in severity. It may be so mild as to give rise to no clinical symptoms. A carrier state has been thought to exist in which, although a person does not have symptoms of Mediterranean disease, he may pass on to his progeny a genetic trait which ultimately may result in a clinically recognizable form of the illness. Thus, Mediterranean disease can exist in a patient of any age, and it may be severe, mild or clinically detectable only by a thorough study of the blood.

Three adult patients who had Mediterranean disease of varying severity were studied at the Mayo Clinic in October of 1946. They were sisters of Italian descent, all 3 at one time or another had had ulcerations of the skin of the legs. A study of the literature on Mediterranean disease has failed to disclose mention of ulcers of the leg occurring in this type of anemia. It is the purpose of this paper to report ulceration of the skin of the legs as a manifestation of Mediterranean disease. Each of these patients stated that she had been anemic many years, and that a diagnosis of Cooley's anemia had been made many times.

The first patient was a 30 year old Italian woman who complained of soreness in the right upper abdominal quadrant. She had been informed by her local physician that she had gallstones. When she had been 14 years old, ulceration of the skin over the medial aspect of the left ankle had developed. This had lasted about a year and had healed slowly, leaving a scar. During the general physical examination moderate pallor of the oral mucous membrane was seen. There was tenderness to deep palpation in the right subcostal region, and the tip of the spleen could be felt 4 cm. below the left costal margin. A small pigmented scar was seen over the medial aspect of the left ankle. There were no other significant physical observations. Except for the ulcers on the leg, this patient had not been incapacitated by her disease.

The second patient was 23 years old. She came to the clinic because of an ulcer of the skin on her right leg. When she had been 12 years of age, two ulcers over the midportion of the right leg had developed. These had lasted for two years and finally had healed, leaving scars. At 15 years of age an ulcer above the right lateral

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malleolus had appeared and had lasted for two years before it healed. At 20 years an ulcer had developed above the left lateral malleolus. This had been treated with balsam of Peru, but at the time of the examination it had not healed. In spite of long-existing anemia, in which the patient said the value for hemoglobin had averaged 8 Gm per 100 cc of blood for many years, she had not been incapacitated except by the ulcers of the leg. During the general physical examination a yellow pallor of the skin was noted. A systolic murmur was heard over the entire precordium. The spleen was palpable 7 cm below the left costal margin, and the liver



FIG. 1. INDOLENT ULCER ON THE LEFT LEG OF A FEMALE PATIENT TWENTY-THREE YEARS OLD WHO HAD MEDITERRANEAN ANEMIA. NOTE THE ATROPHIC SCARS AT THE SITES OF OLD HEALED ULCERS.

was enlarged to a point 3 cm below the right costal margin. An ischemic-appearing indolent ulcer 4 cm in diameter was present on the lateral surface of the left leg above the external malleolus (fig. 1). The border of this ulcer was irregular, but was not undermined. There was considerable hyperpigmentation at the periphery of the ulcer, but in this zone there was only minimal cutaneous sclerosis. The base of the ulcer was clean. Three scars, the sites of previous ulceration, were noted (1) on the right leg, (2) on the anterior surface of the left leg, in its upper third part and (3) on the lateral surface of the left leg. These scars had a hyperpigmented periphery and a glossy, atrophic center. There were no other significant physical observations.



The third patient was 19 years old. She came to the clinic because she had an ulcer on her right ankle which had remained unhealed for a year. When she had been 17 years old an ulcer on the left ankle had developed which had been treated with penicillin without apparent effect. This ulcer had healed slowly over a period of eighteen months. A year later an ulcer on the right ankle had appeared and had been treated with balsam of Peru, but it had not healed.

During the general physical examination pallor of the mucous membranes and yellow pallor of the skin were noted. The heart was enlarged and the apical impulse could be palpated at the anterior axillary line. A loud blowing systolic murmur was heard over the entire precordium, and it was maximal at the cardiac apex. Dyspnea was evident when the patient was at rest, and was pronounced after any exertion. The spleen extended to the umbilicus, and the liver was palpable 7 cm below the right costal margin. A triangular ulceration of the skin 1.5 cm in diameter was noted just distal to the right external malleolus. This ulcer was similar to the one described in the second patient. The present patient was suffering from a severe form of Mediterranean disease, and was greatly handicapped.

In none of these patients was there evidence of arterial or venous insufficiency in the extremities. Local treatment for the ulcers and the administration of iron, liver extract and whole blood had not brought about any improvement in the ulcers or the general health of the patients. In roentgenograms of the skull, only those of the third patient demonstrated changes of significance. General osteoporosis, with thinning of both the inner and outer tables of the skull, was noted.

In smears of specimens of blood from all 3 patients similar changes characteristic of Mediterranean disease were seen. The changes were of a severity in proportion to the extent of the anemia. These changes consisted of a great variation in the size and shape of erythrocytes, with many microcytes but no spherocytes. Hypochromasia and polychromatophilia were marked, and normoblasts were present. Target cells were seen in all smears, but they were not conspicuous. Myeloid immaturity was not noted.

Studies of sternal bone marrow were made in each case. In all three there was hyperplastic erythropoiesis of the normoblastic type. Megaloblasts were not seen. It may be of some significance that the mature erythrocytes in the marrow smears were of more uniform size and shape than were those seen in specimens of peripheral blood. Pertinent laboratory data are summarized in table 1.

The diagnosis of Mediterranean disease was made in these three cases on the basis of familial anemia, increased resistance of the erythrocytes to hemolysis in hypotonic solution of sodium chloride, very active normoblastic erythropoiesis, and the failure of the anemia to respond to any therapy. No other cause for the anemia could be found. Sick cell anemia was eliminated by the absence of sickling, and congenital hemolytic icterus, had it been present, should have produced spherocytosis and increased fragility of the erythrocytes.

A piece of skin was removed for biopsy from the margin of the ulcer on the leg of the 23 year old patient. Epidermal changes consisted of minimal hyperkeratosis and irregular acanthosis. In the upper half of the cutis the capillaries and arterioles

were increased in size and number, and there was moderate infiltration of lymphocytes, connective-tissue cells, chromatophores and polymorphonuclear leukocytes. In the midportion of the cutis there were several areas of beginning necrosis with

TABLE 1—*Results of Laboratory Studies Three Patients with Mediterranean Disease*

Test	Patient		
	1	2	3
Hemoglobin, grams per 100 cc	10.0	8.1	6.3
Erythrocytes, per cubic millimeter	4,140,000	4,140,000	2,870,000
Leukocytes, per cubic millimeter	10,900	11,500	6,300
Erythrocyte fragility in sodium chloride solution, per cent	42-28*	46-28*	44-28*
Beck and Hertz	No sickling	No sickling	No sickling
Serum bilirubin, milligrams per 100 cc			
Direct	0	0	0
Indirect	1.3	1.3	1.7
Fecal urobilinogen, milligrams (24 hour excretion)	109 169	188 289	223 244

\* Incomplete



FIG. 2. SPECIMEN TAKEN FOR BIOPSY FROM THE MARGIN OF AN ULCER ON THE LEG OF A PATIENT WITH MEDITERRANEAN ANEMIA, SHOWING DENSE DEPOSITS OF IRON IN THE MIDPORTION AND LOWER PORTION OF THE CUTIS (HEMATOXYLIN AND EOSIN  $\times 25$ )

disintegration of cells, so that recognition of cell types in these areas was not possible. Elastic tissue was absent throughout the upper portion of the cutis, and very little elastic tissue was present in the vessels. Degenerative changes, homo-

genization of collagen and moderate edema were seen in the arteriolar walls. In occasional vessels there was proliferative intimal thickening, although in most of the vessels there was no significant alteration of wall-to-lumen ratio. Dense deposits of iron (fig. 2) were seen in the midportions and lower portions of the cutis stained with ferric thiocyanide.

The dense deposits of iron in these sections are of great interest. Whipple and Bradford<sup>2</sup> have studied the deposition of iron-containing pigment in the organs of patients who died of Mediterranean disease. They considered this pigment to be as characteristic of the disease as is any other finding. It resembles that seen in hemochromatosis in adult persons and is seen in most of the organs of the body. Whipple and Bradford made no mention of such deposits in the skin. Mills<sup>3</sup> has reported results of a postmortem study of the skin of a child who died of Mediterranean disease. He considered the pigment he found to be melanin, and not hemosiderin.

When the differential diagnosis of ulcers on the legs of these patients was considered, it was apparent that the lesions were not secondary to occlusive arterial diseases such as thrombo-angiitis or arteriosclerosis obliterans. The absence of hypertension excluded the possibility of the ischemic type of ulceration occasionally associated with hypertension. Venous stasis could not have caused the ulcers because there were no varicose veins and chronic venous insufficiency was not present. Trophic disturbances such as might be caused by syringomyelia, tumor of the spinal cord or tabes dorsalis were excluded. Chronic granulomas as seen in syphilis, tuberculosis, sarcoidosis or dermatomycosis would have produced different histopathologic changes.

The ulcerations of the skin of the legs which occur in sickle cell anemia and congenital hemolytic icterus do not have a distinctive gross appearance which could be used to distinguish them from each other or from those in the patients we are discussing. It seems to us that the chronic ulcer on the leg of an anemic patient is not pathognomonic of a specific type of anemia. The exact character of the anemia must be determined by appropriate clinical and hematologic study.

### SUMMARY

Ulceration of the skin of the legs may occur in Mediterranean disease. Such ulceration cannot be distinguished grossly from that occurring in sickle cell anemia and congenital hemolytic icterus. The outstanding histologic feature (noted at biopsy of one of these ulcers) is the prominent deposition of iron in the cutis.

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## EDITORIAL

### THE HEMOLYTIC CRISIS

THE HEMOLYTIC crisis which occurs so suddenly in cases of familial hemolytic jaundice (familial spherocytosis) has excited interest but its pathogenesis has remained a mystery since it was first described as "crise de déglobulization". Adding to its mystery is its fairly frequent occurrence in successive members of the same family, the cases usually appearing within a few days of each other<sup>1-3</sup>

During the crisis there is a precipitous reduction in the red cell count, accompanied by such constitutional symptoms as fever, vomiting and malaise. Some blood findings have received relatively scant attention, although occasional observers have noted the rather paradoxical reductions in leukocytes, platelets and reticulocytes which may be present—paradoxical because in excessive blood destruction one expects regenerative efforts on the part of the bone marrow with resultant increases in reticulocytes, leukocytes and platelets. What is more, reticulocytosis is considered almost as pathognomonic a finding of the disease as spherocytosis.

Some years ago, I remarked on the pancytopenia which occurred in three cases of familial crisis<sup>1</sup> and stated that "one may speculate as to whether this is due to a hormonal influence of the spleen on the marrow, with the result that the maturation or delivery of cells to the circulation becomes inhibited." In studying a recent case\* Bloom and I found, during the crisis, marked pancytopenia with a complete lack of reticulocytes for four days. Simultaneously, the marrow showed maturation arrest of the nucleated red cells at the primitive or erythrogonic level. When the arrested maturation was spontaneously re-established, reticulocytosis took place in the peripheral blood and the blood counts then returned to pre-crisis levels. We have interpreted the events occurring in crisis as due largely to "hypersplenic" effects with resultant hyperhemolysis, as well as inhibitory effects upon the marrow, the latter resulting in maturation arrest and diminished delivery of marrow cells to the blood.

Owren, whose comprehensive article on the hemolytic crisis appears in this issue, submits a different interpretation for the same set of data. He states that there is no evidence for increased hemolysis during crisis but that the extreme reductions in red cells, leukocytes, platelets and reticulocytes are due to a sudden hypoplastic or aplastic disturbance in the marrow. On the other hand, the extreme spherocytosis of the crisis, the drop in red cell count of from 10 to 30 millions within a day or two of onset and the remarkably quick response in all the various blood cellular constituents which occurs with splenectomy, would seem to argue against Owren's thesis. Nevertheless, Owen's observations are of unusual interest and should serve to provoke further work on the pathogenesis of the crisis, and, indeed, on the fundamental mechanisms of familial hemolytic spherocytosis as well. It is likely that

\* Dameshek, William and Bloom, Marvin S. The Events in the Hemolytic Crisis with Particular Reference to Reticulocytopenia. To be published in one of the forthcoming issues of *Blood* dedicated to Dr. George R. Minot.

two mechanisms, acting simultaneously, are responsible for the extremely rapid drops in red cell count (1) maturation arrest and (2) hyperhemolysis

The development of extreme spherocytosis in the crisis might indicate that this was due to the activity of some extrinsic hemolytic factor acting upon mature red cells, thus causing their rapid destruction. If this is true for the crisis, in which we have occasionally found the presence of a serum auto-hemolysin, it is also a possibility for the less marked spherocytosis and anemia during the long periods between crises. In other words, is congenital spherocytosis really due to a fundamental defect in erythropoiesis as most observers maintain, or is it dependent upon the action of some hemolytic factor which may be specific for the individual's own red cells? In favor of this concept is the relatively large size of the polychromatophilic reticulocytes, which are quite in contrast with the more mature orthochromatic spherocytes. This might indicate that 'hemolysin' has converted normal non-nucleated reticulocytes to injured red cells, i.e. the spherocytes. This would take the disease out of the bone marrow and into some vascular or intravascular site. Be that as it may, there are still many unsolved problems in this, the first described and best known form of hemolytic anemia, and a portent of work to come.

WILLIAM DAMESHEK

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# ABSTRACTS

JOSEPH F. ROSS, M.D., *Editor*

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## ERYTHROCYTES AND ERYTHROCYTIC DISEASE

CHEMICAL FACTORS IN PERNICIOUS ANEMIA *H. K. King* From the Bacteriology Department, Edinburgh University, Edinburgh, Scotland *Edinburgh M. J.* 54: 50-60, January 1947

This article summarizes the many attempts which have been made to assay the antianemic factor in pernicious anemia. The limitations of present knowledge of the intrinsic, extrinsic and liver factors are evident from this discussion. With the impetus provided by folic acid towards the isolation of these factors in their pure chemical state, the article is timely in expressing the need for a satisfactory method other than therapeutic trial in patients for assaying newly prepared compounds. One hundred and one references are cited.

C. A. F.

CASTLE'S TEST IN PERNICIOUS TAPEWORM ANEMIA. *DIPHYLLOBOTRIUM LATUM* AND PERNICIOUS ANEMIA. VII. *B. von Bonsdorff* Helsingfors, Finland. *Acta Med. Scand. Suppl.* 196, Hilding Berglund Anniversary Volume, P. 456-477, 1947.

Tapeworm anemia of pernicious type is the classic problem of Finnish hematology. The author has published a series of very interesting investigations on the gastric secretion in this condition. It has been shown that liver extracts, either intramuscularly or perorally as well as desiccated stomach perorally, are able to induce a remission in this malady. The paper deals with the action of intrinsic plus extrinsic factor in tapeworm anemia. Bonsdorff has already shown that intrinsic factor is present in the gastric juice of these patients. There is no remission if the worm is expelled and the patient is living on a diet free from extrinsic factor (Extrinsic factor however was obviously available in the diets of these anemic patients).

A large series of very thorough experiments were performed and it was found that mixture of extrinsic factor (meat or yeast extracts) and intrinsic factor (human gastric juice) gave no response in tapeworm anemia. The same preparations were active in cryptogenetic pernicious anemia. The results were constantly negative regardless of whether the mixture was incubated for 6 hours before administration or not. The explanation of these data is discussed. It is pointed out that the liver principle itself is not formed *in vitro*. The hypothesis of Formijne that the interaction of the two factors (ex- and in-) takes place in the intestinal wall is discussed. The inhibiting effect of the tapeworm should then occur at this level.

J. W.

THE INTRINSIC FACTOR ACTIVITY OF HIGHLY PURIFIED PREPARATIONS OF AMINOPOLYPEPTIDASE II. *G. Ågren and J. Waldenström* From the Department of Medical Chemistry and the Medical Clinic of the University, Uppsala, Sweden. *Acta Med. Scand. Suppl.* 196, Hilding Berglund, Anniversary Volume, p. 432-455, 1947.

Ågren has pointed out that the intrinsic factor may well be identical with the enzyme aminopolypeptidase that has been purified and investigated by him. Concentrated enzyme preparations were used as a source of intrinsic factor and incubated with beef muscles that had been predigested with pepsin. This preparation seemed to be active. Control experiments were performed on 2 patients with only aminopolypeptidase without beef. In one case the effect as regards reticulocytes and bone-marrow was negative. The serum iron possibly dropped somewhat. The effect of later peroral doses of folic acid was excellent. In the second case there was a definite drop of the serum iron and an irregular increase in

reticulocytes with a normoblastic reaction in the bone marrow. There was no secondary effect of folic acid as regards reticulocytes, but there was a good increase in erythrocytes and hemoglobin. The results tend to show that the enzyme preparation may be active without the presence of extrinsic factor. The authors point out that further experiments are necessary.

J W

ON THE CURATIVE EFFECT OF PURE DUODENAL SECRETION FROM SWINE IN CASES OF PERNICIOUS ANEMIA  
*E. Landboe-Christensen and C. L. S. Bohn* From the Department of Anatomy, Faculty of Medicine, University of Copenhagen. *Acta Med Scand* 127: 116-129, 1947

The authors studied the effect of the juice from a duodenal pouch in swine. This is regarded as the only way of avoiding contamination with gastric secretions. One patient got 150 ml. duodenal juice daily for two weeks. Some pepsin and some HCl were added. The food of the patient was not controlled. There was no immediate increase in reticulocytes but the second response to desiccated stomach also gave very poor results. In the second case 150 ml. pure duodenal juice was given with a noncontrolled food intake for ten days. The reticulocyte response was quick and marked. The bone marrow changed from megaloblastic to normoblastic. Liver extracts gave no secondary reticulocyte reaction. The authors conclude that an antipernicious-anemia-factor is present in the duodenal secretion from swine. They point out that there may be differences between the conditions in man and in animals.

It seems to the reviewer that the effect of purified aminopolypeptidase solutions mentioned in the previous paper and the effectiveness of pure duodenal juice should be further investigated as it may give a clue to the real nature of Castle's principle.

J W

ANEMIE HYPERCHROME AVEC MEGALOCYTES, REFRACTAIRE A L'HEPATOTHERAPIE. ACHRESTIC ANEMIA  
*E. F. J. H. Galger* From the Clinique Medicale de l'Hôpital St. Jan, Zaandam, Holland. *Acta Med Scand* 126: 505-527, 1947

There has been considerable discussion about the real nature of achrestic anemia as described by Wilkinson and it seems as if most continental hematologists regard it as a type of aplastic anemia that has nothing to do with pernicious anemia. The recent successful treatment of such liver refractory cases with folic acid gives this problem more than academic interest. The author is of the opinion that these cases have a really megaloblastic bone marrow in spite of the presence of hydrochloric acid in the gastric juice. There were no symptoms from the nervous system. Potent liver extracts gave no reticulocytosis and no drop in the serum iron. Big transfusions were necessary. Nicotinic acid and yeast extracts were not of any help in the first case. The second one developed anemia during her pregnancy. After delivery the anemia progressed and was not influenced by large doses of different liver extracts. Later there was found an irregular rise in reticulocytes and there was a slow improvement of the blood values. After one year there was still a definite macrocytic anemia but the megaloblastic bone-marrow had become normal. In both cases it seems as if the gastric juice would have been free from intrinsic factor.

Possibly these cases might respond to treatment with folic acid.

J W

PERNICIOUS ANEMIA IN CHILDHOOD. II. RESPONSE TO FOLIC ACID. *J. C. Peterson* From the Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tenn. *Am J Dis Child* 73: 578-580, 1947

The author gives a brief follow-up report on a previously described (*Am J Dis Child* 71: 252-268, 1946) case of pernicious anemia in a 6 year old girl whose history of anemia dated back to the age of 8 months. There was maximal response to administration of liver extract, but continuous therapy was required to maintain a remission. From 1940 to 1946 she was observed during the course of five relapses, some specifically induced by withdrawal of treatment. During such a relapse in 1946 she was given 5 mg. of folic acid orally twice daily. The response to folic acid was similar to that previously observed following the administration of liver extract.

L E Y

THE CLINICAL MANIFESTATIONS OF SICKLE CELL ANEMIA. *V. Grover* Brooklyn, N. Y. *Ann Int Med* 26: 843-851, 1947

This article, which tabulates the signs and symptoms of 48 patients with sickle cell anemia, provides useful data as to the incidence of these various manifestations. Of interest is the height of fever (although

it is not clear whether this is related to incurrent infection or to the sickle cell disease), the frequency of pain in the lower extremities and abdomen, the leg ulceration observed in 25 per cent of the cases, the presence of mental deficiency in 7 cases, and various cardiac manifestations. The authors also remark on the rapid change in size of the spleen and liver over a number of days.

C A F

**LIPID METABOLISM AND DEVELOPMENT OF ANEMIA IN SPLENECTOMIZED GUINEA PIGS FED CHOLESTEROL**  
*B Kennedy and R Okey* From the Department of Home Economics, University of California, Berkeley  
*Am J Physiol* 149 1-6, 1947

Guinea pigs fed diets containing 1 per cent cholesterol and 15 per cent fat develop, in chronologic order, fatty livers rich in cholesterol, splenomegaly, and severe anemia in which the red cells are both excessively fragile and excessively resistant to hypotonic solutions. The resulting syndrome is a hemolytic anemia and includes anemia, hyperbilirubinemia, bone marrow hyperplasia, pigment gallstones, and hemosiderosis. This disease is frequently cured by splenectomy. The authors wondered whether the anemia could, therefore, be due to the splenic hyperplasia, *c g*, an enlarged spleen excessively destroying red cells.

They therefore first splenectomized guinea pigs and then, after a control period of waiting, fed them cholesterol and fat. The resulting animals lost weight and developed anemia with hemolysis just as the original guinea pigs. The liver and blood lipids, and the incidence of gallstones, were identical in splenectomized and intact guinea pigs. The conclusion was therefore reached that the spleen was not primarily responsible for the anemia.

A parallel may be drawn to the congenital spherocytosis of human beings, in which splenectomy, by removing the chief site of red cell destruction, 'cures' the hemolytic syndrome, although the fundamental aberration (spherocytosis) remains. It seems more than ever advantageous to divide hemolytic anemias into three types: (1) intrinsic defects of the red cells, making them especially vulnerable to the normal action of the spleen, (2) action of demonstrable or nondemonstrable plasma or tissue substances on originally normal red cells (*c g*, circulating agglutinins). The guinea pig anemia probably fits best here. Such damaged red cells are also particularly liable to destruction, especially in the spleen, and (3) excessive destruction of normal red cells by a hyperactive spleen (hypersplenic hemolytic anemia). In all three types, splenectomy may be curative, but it is only in the relatively rare third category that the spleen is fundamentally responsible for the disease.

S E

**THE USE OF FOLIC ACID IN SPRUE** *R M Suárez, T D Spies, and R M Suarez, Jr* From the School of Tropical Medicine of Puerto Rico, San Juan Puerto Rico, and the University of Cincinnati, Cincinnati, Ohio  
*Ann Int Med*, 26 643-677, 1947

This is a comprehensive report on the management of 50 cases of tropical sprue with folic acid. Twenty-two cases were acute and severe. These cases were thoroughly studied with sternal marrow differential counts, complete hematologic studies of the peripheral blood, blood chemistries, gastric analyses, stool urobilinogen, and the fat content and fat partition of the stools. Of particular interest are the changes in stool, glucose tolerance tests and hematologic picture after treatment. Admission stools on 12 patients averaged 19.18 per cent solid material in fresh feces, 26.46 per cent total fat in dried feces, 79.31 per cent free fatty acids and 85.22 per cent of total split fats. A few days after treatment the solid content of feces was 13.19 per cent, and there was 20.76 per cent total fat in dried feces, 67.33 per cent being free fatty acids and total split fat being 72.75 per cent. (Normal values for solids 25.23 per cent, total fats in dried feces 21.23 per cent, free fatty acids 66.77 per cent, and total split fat 72.28 per cent.) Oral glucose tolerance tests before treatment showed an increase in blood sugar of between 1 and 32.5 mg per cent. After 1 to 2 months treatment all but 2 showed improvement and 6 of 14 showed a rise of greater than 40 mg per cent.

Ten cases were summarized with a discussion of reticulocyte and red cell responses to varying doses of folic acid. The patients were maintained on either a diet poor in proteins or a sprue diet rich in these materials. On the poor diet hematologic response was submaximal even with doses up to 100 mg of folic acid. With an adequate diet a daily dose of 20 mg of folic acid by mouth gave a good initial response. The authors state that the patients are maintained on as little as 2½ to 5 mg per day. Follow-up on these patients extended over only 1 to 4 months.

C A F



**SYNTHETIC FOLIC ACID THE EFFECTIVENESS OF A CONJUGATED FORM IN THE TREATMENT OF TROPICAL SPRUE, ADDISONIAN PERNICIOUS ANEMIA AND NUTRITIONAL MACROCYTIC ANEMIA** *T D Spies, G G Lopez, F Mslanes, and T Aramburu* From the University of Cincinnati at Hillman Hospital, Birmingham, and the Calixto Garcia Hospital and the University of Havana, Cuba *J A M A* 134 18-22 1947

This is another report on the clinical efficacy, in the treatment of macrocytic anemias, of compound similar chemically to folic acid. To date, these substances can be classified into three groups (1) pteroyl glutamic acid, which is the compound found in liver L casei factor, in yeast L casei factor, and vitamin B<sub>12</sub>, (2) pteroyl-diglutamyl glutamic acid, which is the fermentation L casei factor, and (3) pteroyl hexaglutamyl glutamic acid, which is the "vitamin B<sub>12</sub> conjugate" material. The chemical difference among these substances lies in the number of glutamic acid residues per molecule.

The present report relates the response of patients with macrocytic anemias to pteroyl-diglutamyl glutamic acid, with the usual reticulocytosis and subsequent clinical and laboratory improvement similar response to pteroyl-glutamyl glutamic acid (2 glutamic acid residues) has previously been published (T D Spies, R E Stone, and R L Toca, *South Med J* 40 175-6, 1947).

The authors suggest that the more complicated chemical compounds, such as the hexaglutamyl compound of vitamin B<sub>12</sub> conjugate, may be broken down with difficulty by some patients with macrocytic anemias. The relative efficacies of these and other similar compounds may ultimately help to elucidate their manner of action in pernicious anemia, and perhaps to discover an ideal substance for clinical use.

S E

**THE BLOOD PICTURE AND PLASMA PROTEIN LEVEL FOLLOWING INJURY** *J Vaughan, M Thomson and M Dyson* From the N W London Blood Supply Depot, London, England *J Path & Bact* 58 749-763 1947

Under very trying war-time conditions the three groups of patients studied consisted of (1) accident other than those due to enemy action, (2) industrial accidents in which superficial burns were associated with fractures, and (3) accidents due to enemy action. The results of the various determinations showed that both erythrocyte and hemoglobin values fall during the first week and then rise to a higher level. Reticulocytes were low immediately following the injury, but rose to the upper limit of normal immediately preceding the rise in hemoglobin. The elevated sedimentation rate was very likely due to the increased plasma fibrinogen. Although the leukocytes decreased slightly during the period of observation, they remained within normal limits. Leukopenia was not encountered.

O P J

**THE EFFECT OF DIFFERENT DIETS AND OF IRON MEDICATION ON THE NUTRITIONAL ANEMIA OF INDIAN ARMY RECRUITS** *M Hynes, M Ishaq, and O P Verma* From the Anemia Investigation Team, Medical Directorate, General Headquarters, India. *The Indian J M Research* 34 273-288, 1946

In a preceding report this team of investigators had shown a correlation between the presence of anemia and evidence of malnutrition as indicated by muscular development and dermal and ocular signs of vitamin deficiency. There was found to be a direct correlation between the hemoglobin level and the cell volume and mean corpuscular hemoglobin concentration so that all severe or moderately severe anemias were microcytic and hypochromic. It was noted that the amount of hookworm infection could be correlated with the severity of the anemia in only the more malnourished third of the recruits.

During the first six months after induction there was a rise in the hemoglobin level in the group that was given the army diet alone, but the most severe anemias did not reach a normal level at the end of six months. It was found that three grains of ferrous sulfate daily cured all the anemias at the end of four months and was judged to be as effective as six grains daily. The rate of hemoglobin rise was less in both the treated and control group if milk was substituted for meat as the source of protein in the diet. It was found that hookworm infection did not interfere with the rate of hemoglobin regeneration but that septic infections of the areolar tissue, tonsillitis and malaria depressed hemoglobin regeneration. This is an excellently controlled mass study of the effect of iron therapy and gross alterations in the diet on hemoglobin regeneration in human subjects.

R S E

LES MALADIES ERYTHREMIQUES (ERYTHREMIC DISEASES) *G. Di Guglielmo*, Directeur de l'Institut de pathologie spéciale de Naples (Italy) *Rev. Hémat.* 1: 355-398, 1946

This important study is founded on 80 observations of erythremia (Erythroleukemia) most of them published in Italy and in particular by Di Guglielmo who was the first to describe the disease in 1917.

The author defines the disease as follows: Generalized primitive proliferation of the erythroblastic series, which may be acute or chronic, occur in all ages without sex preference. The acute form, the less rare, was the first to be known. The symptoms are the following: Anemia, fever, splenomegaly and hepatomegaly. In the blood young erythroblasts are seen, which is often atypical, and there is in the erythropoietic center an embryonic proliferation of erythroblasts. The evolution lasts only a few weeks. The chronic form is also marked by anemia and hepato-splenomegaly, but the erythroblasts found in the peripheral blood are more mature forms. The duration of this form is about two years.

In both forms, the anemia is a constant feature, and always severe. The mechanism is complex: lack of formation of normal erythroblasts and increased destruction of abnormal red cells in peripheral blood. The author supplies rich pathologic material from biopsies and postmortem examinations (illustrated by 28 microphotographic and color plates). There is a striking erythroblastic proliferation and an important reticulo-endothelial proliferation in the bone marrow, lymph nodes, liver, and also in the kidneys, heart, pancreas, and adrenals. A complete bibliography terminates this weighty study.

J P S

INHERITED DISORDERS OF THE BLOOD IN RODENTS *H. Grüneberg* *Proc. Roy. Soc. Med.* 40: 191-192, 1947

The author of this short note has done a large amount of work on hereditary mechanisms of various anemias in the rat and the mouse. Most of the work is not obviously related to clinical mechanisms, but relationships between certain of these disorders and certain anemias in man have been suggested.

Seven disorders are discussed by Grüneberg. Among these several are of special interest. Siderocytic anemia of the mouse is associated with a recessive gene for certain other characteristics, the characteristic finding is the presence of nonhemoglobin iron in the erythrocytes. Grüneberg conceives of these cells as aged cells, counterparts of the reticulocytes, and notes that they are present in various human hemolytic anemias in proportion to the severity of the hemolysis.

A lethal spherocytic anemia occurs as a recessive characteristic in the rat which Grüneberg attributes to a faulty marrow which produces red cells of inferior quality, whose survival time in the circulation is reduced. This anemia is not analogous to familial spherocytosis of human beings. A second form of familial hemolytic anemia, however, occurs as the recessive acholuric jaundice of the rat. Homozygotes have microcytosis, increased hypotonic fragility, jaundice, and splenomegaly. Heterozygotes may show no abnormality, or may show increased hypotonic fragility and reticulocytosis without jaundice. The possible relationship of this disorder, whose heredity has been worked out, to familial spherocytosis of humans is intriguing. The disease in the rat, however, is not cured by splenectomy, so that a definite parallel cannot be drawn.

As in other fields in medicine, study of disorders occurring in animals breeding under controlled laboratory conditions may be expected to shed light on similar disorders in the clinic. The use of known hereditary mechanisms in rodents to elucidate the mechanisms of similar human disorders is another line of attack in these still puzzling problems.

S E

HAEMOLYTIC ICTERUS (ACHOLURIC JAUNDICE), CONGENITAL AND ACQUIRED *J. F. Loutit and P. L. Mollison*

From the South London Blood Supply Depot, London, England *J. Path. & Bact.* 58: 711-728, 1947

Hemolytic anemias have been the center of much controversy ever since they were first studied. The present article contains evidence which indicates that congenital and acquired cases of hemolytic icterus are both physiologically and serologically different from each other. They are two etiologically different conditions within the syndrome of acholuric jaundice. Some of the evidence in support of this belief is that when normal blood is transfused to recipients with the congenital type of anemia, the survival of these cells is from 100-120 days. But when recipients with the acquired type of anemia are transfused with normal blood, the survival of the cells is markedly reduced. In addition to this, when washed red cells from cases of congenital acholuric jaundice are tested for agglutination with anti-human-serum serum they are not agglutinated, but cells from cases of acquired acholuric jaundice readily agglutinate. Loutit

and Mollison believe that the congenital type of hemolytic icterus is due to a hereditary defect of the erythroid elements and that in the acquired type an abnormal hemolysin co hemolysin system is in action

O P J

FURTHER STUDIES ON THE RELATIONSHIP BETWEEN CELL PERMEABILITY AND METABOLISM THE EFFECT OF CERTAIN RESPIRATORY INHIBITORS ON THE PERMEABILITY OF ERYTHROCYTES TO NON-ELECTROLYTES  
*F R Hunter* From the Department of Zoology, University of Oklahoma, Norman, Oklahoma *J Cell & Comp Physiol* 29 301-312, 1947

The purpose of the experiments reported in this paper was to determine whether or not selective permeability depends on energy derived from metabolic activities within the cell. For this purpose beef and chicken blood was used. Substances, such as potassium cyanide, sodium fluoride, arsenious oxide and iodoacetate, which interfere with metabolic activities, were allowed to act on blood corpuscles at  $37^{\circ} \pm 0.1^{\circ} \text{C}$  for varying periods of time. Although metabolic systems were sensitive to these substances and inhibited by them, there was not a change in the permeability. Apparently the permeability of the erythrocyte to non-electrolytes does not depend on energy derived from either aerobic or anaerobic metabolism.

O P J

THE EFFECT OF WASHING ON THE PERMEABILITY AND METABOLISM OF CHICKEN ERYTHROCYTES  
*F R Hunter* From the Department of Zoology, University of Oklahoma, Norman, Oklahoma *J Cell & Comp Physiol* 29 313-321, 1947

In the previous paper, metabolic activities of erythrocytes were inhibited by the addition of various substances. In the present paper, similar activities have been influenced by removing certain components of the enzyme-substrate by washing chicken erythrocytes three or four times with Ringer-Locke solution. In so doing, anaerobic glycolysis was reduced to an immeasurable level. Unhemolyzed cells were present after they had been exposed to anaerobic conditions for as long as 384 hours. In these experiments, it was necessary to avoid contamination with hemolytic bacteria. The results of these experiments show that permeability of the chicken erythrocyte to glycerol does not depend on energy derived from cell metabolism.

O P J

## IMMUNOHEMATOLOGY

DEMONSTRATION OF ANTIBODIES IN ACQUIRED HEMOLYTIC ANEMIA WITH ANTI-HUMAN GLOBULIN SERUM  
*R S Evans, R T Duane and V Behrendt* From the Department of Medicine, Stanford University School of Medicine, San Francisco, Calif *Proc Soc Exper Biol & Med* 64 372-375, 1947

The Coombs test which determines the red cell agglutinating effect of serum from rabbits immunized against human serum globulins, was applied with positive results in two cases with acquired hemolytic anemia, indicating the presence of an adsorbed immune body type of hemolytic agent attached to their erythrocytes. Normal blood transfused into these patients shared in the hemolytic process, presumably as a result of exposure of the donor cells to, and their adsorption of, a hemolytic agent present in the circulation of the recipients. It was not possible to demonstrate any hemolytic properties, or adsorbable antibodies in the serums of these patients, which may be explained on the basis of a continuous excess of antigen provided by the circulating red cell mass. Support for this thesis was obtained from experiments in which the immune substance, probably the active anti-red cell antibody, was eluted in warm saline from the affected red cells of the patients, and then combined, *in vitro*, with normal cells.

C P E

ÉTUDE SUR L'ICTÈRE HÉMOLYTIQUE EXPÉRIMENTAL PAR INJECTION ET INGESTION D'ANTISÉRUM (STUDY ON EXPERIMENTAL HAEMOLYTIC JAUNDICE, BY ANTISERUM INJECTION OR INGESTION)  
*M Bessis and P Freixa* Laboratoire de Recherches du Centre National de la Transfusion Sanguine (Paris) *Rev Hemat* 2 114-146, 1947

The injection of anti-red cell serum into the rat provokes an acute syndrome with hemoglobinuria, an acute hemolytic anemia, or a subacute curable anemia. These experiments with rats bring confirmation

to the Danieshek and Schwartz experiments with guinea pigs, and also show that increased osmotic fragility and microspherocytosis is easily induced. The personal contribution of the authors is as follows:

1. In a fresh preparation of red cells one notes a pseudo-agglutination of the spherocytes instead of normal rouleaux-formation. There are some spindle-shaped red cells and numerous erythroblasts. In the acute cases hemoglobin crystals may be seen in the cells.

2. There is a marked leukocytosis with a histiocytic reaction and many leukocytes show erythrophagocytosis. Jaundice was present in subacute or acute form and when it was especially severe erythrophagocytosis was striking.

3. The histologic findings in liver, spleen, and kidneys are identical with those found in hemolytic disease of the human new-born, but nuclear jaundice was not encountered.

4. The causal antibody was found on the red cells of the injected animal (conglutination method).

5. The peroral administration of the anti-serum to the new-born rats induces trouble only if it has been given before the twentieth day.

The authors discuss the relationship between this experimental anemia and the human erythroblastosis of the new-born. This weighty study is illustrated with numerous microphotographs and colored plates (18 pictures).

J P S

**INHIBITION BY CERTAIN POLYSACCHARIDES OF HEMAGGLUTINATION AND OF MULTIPLICATION OF INFLUENZA VIRUS.** *R. H. Green and D. W. Woolley.* From the Laboratories of the Rockefeller Institute for Medical Research, New York, N. Y. *J. Exper. Med.* 86: 55-64, 1947.

The complex carbohydrates, apple pectin, citrus pectin, flaxseed mucilage, blood group A substance, gum acacia and gum myrrh, as well as an aqueous extract of chicken erythrocytes, were shown to inhibit the agglutination of chicken red cells by the PR8 strain of influenza A virus. Many, but not all, of the inhibitory substances were polysaccharides rich in galacturonic acid. Alginate acid, a polysaccharide largely composed of mannuronic acid units, was inactive, as were the simple carbohydrates such as galactose, galacturonic acid and aldobionic acid. Detailed study of the action of apple pectin showed that this substance affected both virus and red cell and that it also inhibited the multiplication of virus in embryonated eggs.

These observations serve well to illustrate the usefulness of the erythrocyte as a tool for the study of virus-cell relationships. The results obtained also illustrate how antagonism between structurally similar compounds may be used as a guide in investigating the biology of viruses.

L E Y

**THE NATURE OF NON-SPECIFIC INHIBITION OF VIRUS HEMAGGLUTINATION.** *W. F. Friedewald, E. S. Miller, and L. R. Whitley.* From the Department of Bacteriology and Immunology, Emory University School of Medicine, Atlanta, Georgia. *J. Exp. Med.* 86: 65-75, 1947.

Saline extracts of lung, liver, kidney and spleen from human beings, rabbits and guinea pigs were found to inhibit hemagglutination by mumps virus and by the PR8 and Lee strains of influenza virus. The inhibition titers of organ extracts were usually higher than the titers obtained with sera from the corresponding animals.

Saline extracts of human and chicken erythrocytes also contained an inhibitory substance in high titer, and these cells were markedly agglutinated by influenza and mumps viruses. Rabbit cells were not appreciably agglutinated by these viruses and extracts of rabbit cells were not inhibitory. Sheep red cells varied in their capacity to agglutinate and also in their yield of inhibitory substance. When the virus receptor substance was removed from chicken cells by adsorption and elution with influenza virus, extracts of the cells were no longer inhibitory.

Evidence is presented that the inhibitory substance is not an antibody and that it is distinct from the blood group factors A, B, and Rh. The findings with the virus inhibitory substance are, nevertheless, compared by the authors to those with A and B factors. Both are found in many types of mammalian cells in addition to erythrocytes and also in various body fluids. The A and B factors, moreover, may combine with isoantibodies to inhibit hemagglutination.

Failure of the inhibitory substance to neutralize influenza virus *in vivo* suggests that it is not an important factor in preventing infection. On the contrary, it is postulated that it may actually have a deleterious effect in human infections by preventing union of virus with antibody.

A recent paper by Bovarnick and de Burgh (*Science* 105 550-551, 1947) should also be cited here. These authors prepared lipid extracts of erythrocytes which inhibited agglutination of these cells by influenza and mumps viruses.

L E Y

THE INCIDENCE OF THE "DANGEROUS" GROUP O DONOR. RESULTS OF THE TITRATION OF ONE THOUSAND CONSECUTIVE SERA. A. Zoutendijk. From the South African Institute for Medical Research, Johannesburg. South African M J 21 438-441, 1947.

Sera from 1000 unselected European group O donors were titrated at 37°C with 2 per cent suspensions of fresh A and B cells. End points were read grossly after incubation for one hour and titers were expressed in terms of the dilution of serum itself and not of the final mixture of serum and cell suspension.

Taking a titer of 1/200 as 'dangerous,' 221 of the donors tested were classified as unsafe for transfusing patients belonging to groups other than O. Of these, 95 had high anti-A titers only, 62 had high anti-B titers only, and 64 of the 221 donors had both high anti-A and anti-B titers. The incidence of high titers is considerably lower in the experience of other investigators, probably due mainly to differences in technique.

The author urges that use of the term, 'universal donor,' be discontinued because it gives a false sense of security. He further recommends that all group O donors be classified according to isoagglutinin titer—a task which is considered reasonable since variations in titer from time to time are small in healthy persons. This procedure is preferred to that of adding A and B group specific substances to O blood prior to transfusion to A, B, or AB recipients.

Although no attempt is made in this paper to discuss the controversial issues involved in the use of universal donors, a timely warning is given.

L E Y

FALSE POSITIVE TESTS FOR SYPHILIS. A STUDY OF THEIR INCIDENCE IN SPOROZOITE-INDUCED VIVAX MALARIA. C R Rein and J F Kent. From the Division of Serology, Army Medical School, Washington, D C. J A M A 133 1001-1003, 1947.

Ninety volunteers were inoculated with *P. vivax* sporozoites in order to study the treatment of the disease. A battery of seven diagnostic tests was done for syphilis, including, in addition to the usual tests, a special test with cardiolipin antigen devised by Rein and Bossack. The following results were obtained:

1. 63.3 per cent of the subjects developed false positives with one or more tests at some time during the course of the malaria.

2. In no instance was there a positive test before the fever or the parasitemia.

3. The false positive test appeared on the average a little over eight days after the parasitemia (range 0-30 days).

4. The false positive test was usually transitory and of low titer. Its duration after a given attack of malaria was from 2 to 98 days, although in one patient who had successive relapses the false positive serologic reaction was present for 517 days.

5. The Kahn standard tests gave the most false positives. The Hinton flocculation test gave the least false positives. The new Rein-Bossack cardiolipin test, a microfloculation procedure, was also seldom falsely positive.

S E

PATHOGENESIS OF PASSIVE RH ISOSENSITIZATION IN THE NEWBORN (ERYTHROBLASTOSIS FETALIS). R R Darrow and J Chapin. From the Women and Children's Hospital, Chicago, Ill. Am J Dis Child 73 257-278, 1947.

The authors advance the hypothesis that Rh antibodies produced as a result of isoimmunization during pregnancy are of two types: erythrocyte-destroying antibodies and sensitizing antibodies. The former act upon the infant's Rh-positive erythrocytes in such a way that their destruction by phagocytosis is greatly accelerated. The sensitizing antibodies, according to the concept presented, exert their effect within tissue cells by reacting with Rh antigen freed by the destruction of Rh-positive erythrocytes. The intracellular reaction injures the cell and causes the release of histamine and possibly other toxic substances into the circulation. These substances may then have a damaging effect on susceptible tissues elsewhere in the body.

It is emphasized that maternal antibodies are carried first to the fetal liver, and that hepatic cells suffer most from the sensitization reaction. Jaundice due to rapid destruction of erythrocytes is intensified by hepatocellular injury, and liver damage is also held at least partly responsible for hemorrhagic diatheses and hypoproteinemia. Edema is attributed to hypoproteinemia and increased capillary permeability, the latter being caused by direct sensitization of the endothelium or by the action of histamine produced in organs such as the liver and lung. It is further suggested that histamine may be responsible for the development of pulmonary edema in the newborn and also for intrauterine asphyxia as a result of placental edema. Possible causes of brain injury are anoxia, intoxication (histamine) and direct sensitization of nuclear neurons. Kernicterus is attributed to the increased avidity of affected cells for bilirubin.

Three of the 5 erythroblastotic infants described were transfused with Rh-positive blood. It is suggested that the favorable results obtained were due in part to the 'desensitizing' action of the Rh-positive cells.

The speculations set forth in this paper are supported to only a limited extent by the authors' personal observations. They should, nevertheless, serve to stimulate further investigation of hemolytic mechanisms, of the much-debated 'toxic' effects of rapid red cell destruction, and the merits of exchange transfusions. Until more is known concerning the pathogenesis of erythroblastosis fetalis, most clinicians will probably prefer to continue their current practice of transfusing affected infants with Rh-negative blood.

L E Y.

LÉSIONS DU SYSTÈME NERVEUX CENTRAL DANS DEUX CAS D'ICTÈRE NUCLÉAIRE DU NOUVEAU-NÉ—(CENTRAL NERVOUS SYSTEM INJURIES IN TWO CASES OF NUCLEAR JAUNDICE IN NEWBORN) *Ivan Bertrand* (Paris)  
Rev d hemat 1 399-420, 1946

The author makes a minute study (topographic and histologic) of two cases of nuclear jaundice due to Rh immunization. His conclusions are the following:

There is a very important extension of the histologic lesions which largely exceeds the macroscopic topography of the biliary impregnation. There is an edematous infiltration of nervous elements and also of the perivascular areas. The neuroganglionic degeneration is characterized by acute tumefaction, liquefaction, and *Schwererkrankung* of Nissl. The cerebral cortex, the paraventricular nuclei of the cerebral trunk, the olives bulbaires and the *noyau dentelé* are the more affected areas. All these lesions are degenerescences without much satellitic reaction (16 pictures and microphotographs).

J P S

AUTOHEMAGGLUTINATION AND RAYNAUD'S PHENOMENON *G B Forbes* Brit M J 1 598-600, 1947

The literature dealing with cold agglutinins is briefly reviewed. Eighteen published case reports of the association of autohemagglutination with peripheral vascular phenomena simulating Raynaud's disease are cited. The author presents clinical and laboratory studies of a case with symptoms of Raynaud's disease caused by the presence of an autohemagglutinin activated by exposure to cold. The titer of this agglutinin was 1,024. There was no predisposing disease to account for the development of this agglutinin.

J F R

HEMOLYTIC TRANSFUSION REACTIONS *M J Nicholson* From the Department of Anesthesiology, Lahey Clinic, Boston, Mass. Lahey Clin Bull 5 101-113, 1947

The author presents a concise review of the subject and properly stresses the importance of (1) clerical errors, (2) masking effect of anesthesia, (3) examinations for presence of hemoglobinemia and hemoglobinuria, and (4) recheck of crossmatch with fresh specimens from donor and recipient.

Of particular interest with regard to therapy is the reference to Diamond's suggested administration of A and B group specific substances to group O patients who have been transfused with group A or group B blood. The object of this procedure is to neutralize the alpha and beta agglutinins in the recipient's serum and thus delay the destruction of remaining transfused incompatible cells.

A case is reported in which a group O Rh positive male was given 500 cc of group A Rh positive blood. The patient made a satisfactory recovery following administration of alkali, group O blood and 40 cc of a solution of A and B factors.

L E Y

## LEUCOCYTES AND LEUCOCYTIC DISEASE

**ALUEKEMIC MYELOSIS CHRONIC NONLEUKEMIC MYELOSIS, AGNOGENIC MYELOID METAPLASIA, OSTEOSCLEROSIS, LEUKO-ERYTHROBLASTIC ANEMIA AND SYNONYMOUS DESIGNATIONS** *E L Heller, M G Lewisohn and W E Palm* From Department of Pathology, University of Pittsburgh and the Presbyterian Hospital, Pittsburgh, Pa *Am J Path* 23 327-365, 1947

It is well known that patients with myelogenous leukemia may differ markedly from one another with respect to their quantitative as well as their qualitative blood pictures. There has been a question as to whether or not some cases are permanently aleukemic. The present article reviews the literature and offers 3 new cases in support of the thesis that there is a disease, aleukemic myelosis, which is fundamentally leukemic in nature. The peripheral blood may contain immature myeloid cells but not in the same degree as a frank leukemia. On the other hand, it may not present a leukemoid reaction. Tissue responses in these patients have been interpreted to indicate a local origin of leukemic cells from the reticulo-endothelial system rather than a metastatic one.

O P J

**THE FURTHER EFFECT OF LEUKOCYTOSIS—PROMOTING FACTOR OF EXUDATES WHEN INJECTED IN CONNECTION WITH INFLAMMATION** *V Menkin* From Chase Foundation for Cancer Research, Temple University School of Medicine, Philadelphia, Pa *Arch Path* 43 566-569, 1947

This is a continuation of the many studies on inflammation which the author has been conducting in recent years. When dogs were caused to develop acute pleurisy by intrapleural injections of turpentine, the leukocyte count returned to normal in about one day. The leukocyte count responded in a similar manner when single intravascular injections of leukocytosis-promoting factor were administered to healthy dogs. But when leukocytosis-promoting factor was administered to dogs with an acute pleurisy, the leukocyte count reached a higher level and remained there for about nine days. The clinical implications are that natural leukocytosis might be reinforced even during antibiotic therapy.

O P J

**GLYCOGEN CONTENT OF ISOLATED WHITE BLOOD CELLS IN GLYCOGEN STORAGE DISEASE** *R Wagner* From The Boston Floating Hospital and the Department of Pediatrics, Tufts College Medical School, Boston, Mass *Am J Dis Child* 73 559-564, 1947

The glycogen concentration in whole blood, plasma, red cells, and in isolated leukocytes was determined repeatedly in a case of the hepatic form of glycogen storage disease (von Gierke's disease). In no case could glycogen be demonstrated in the erythrocytes. Glycogen determinations in whole blood were considered unsatisfactory technically and of limited diagnostic value. In contrast to the plasma of normal persons, which was always found free of glycogen, the plasma in the case described contained 8.7 to 15.6 mg per 100 cc. Determinations of glycogen in isolated leukocytes were considered of greatest diagnostic value. In normal blood the granulated white cell, which is the only carrier of glycogen, was calculated to contain this substance in an average concentration of 4.23 micrograms per million cells. In the case of glycogen storage disease studied, the range of glycogen concentration per million adult polymorphonuclear cells was 2.7 to 7.4 times greater than in normal leukocytes. Evidence is cited that granulocytes are incorporated in the system of tissues serving carbohydrate metabolism.

L E Y

**THE EFFECT OF AGE ON THE LEUKOCYTE COUNT. A COMPARISON OF WHITE BLOOD CELL COUNTS IN AGED AND YOUNG INDIVIDUALS, WITH SPECIAL REFERENCE TO THE RESPONSE TO INFECTION** *W R Galbert, L W Hutaff, and G T Harrell* From the Bowman Gray School of Medicine, Winston-Salem *Geriatrics* 2 96-100, 1947

Since aging results in a deterioration of various systems of the body, a wearing-out process might be expected in the bone-marrow with resultant changes in the values of the circulating blood elements. It might also be expected that changes in the white blood cells might interfere with the anti-infective powers of the patients. This article reports hematologic studies on hospitalized patients of various ages, with analysis of the counts for various age groups.

It was found that the mean white blood count was the same at various ages from 20 to 49 as after the

age of 50 years. It was found also that the response of the white cell count to infection was the same in all decades. These negative results suggest that there is little "wearing-out" of the hematopoietic system in the course of the usual adult life.

S E

**INFECTIOUS MONONUCLEOSIS. AN AUTOPSY REPORT** *F H Allen, Jr and A Kellner* From the Blood Grouping Laboratory, Children's Hospital, Boston. *Am J Path* 23: 463-477, 1947

Although Downey and his associates have divided the leukocytoid lymphocytes found in infectious mononucleosis into three main types, it has long been recognized that there is considerable pleomorphism within a given category. The qualitative blood picture not only varies among individuals but also within a given patient, depending upon the severity and stage of the disease. In like manner, the clinical features of this disease have been characterized as protean. The present article deals with the autopsy findings in a 23 year old American Army Air Forces pilot, who was killed in an accident following a previous acute illness. The diagnosis was established by the clinical picture, hematologic findings and strongly positive heterophil antibody tests. At autopsy focal cellular infiltrations were found in the liver, kidneys, heart, lungs, adrenals, testes and brain. From this it became apparent that infectious mononucleosis is a generalized disease with changes in almost every organ in the body.

O P J

**SPONTANEOUS RUPTURE OF THE SPLEEN DUE TO INFECTIOUS MONONUCLEOSIS** *J M Sullivan and S E Wasserman* From the Veterans Hospital, Wood, Wisconsin, and the Marquette Univ. School of Medicine, Milwaukee, Wisconsin. *J A M A* 134: 144-145, 1947

An additional case of rupture of the enlarged spleen of infectious mononucleosis is reported. The patient was a 25 year old man whose immediate complaints were headache, malaise, weakness, sore throat, and upper abdominal pain. Before admission to the hospital, the patient had sudden agonizing left upper quadrant pain followed by fainting, and several days later developed tenderness and rigidity in the left upper quadrant, pain in the left shoulder, and signs of consolidation or fluid at the left chest. The liver and spleen were not palpable. A blood count was normal. Exploration revealed 1500 cc. of free blood in the abdomen, and two lacerations of the spleen with corresponding subcapsular hematomata. The spleen showed only hyperplasia (weight 695 grams). Blood studies showed abnormal cells on only one occasion, but the heterophile antibody test was positive in a dilution of 1:248 six days after operation. The patient did well.

The authors note that 12 cases of such rupture have been reported from 1944 to 1946.

S E

## BLOOD COAGULATION AND HEMORRHAGIC DISEASES

**THE INTERACTION OF PROTHROMBIN A AND B** *F L Munro and M P Munro* From the Department of Medicine, Jefferson Medical College and Hospital, Philadelphia, Pa. *Am J Physiol* 149: 95-99, 1947

Quick's hypothesis that prothrombin is a complex consisting of two substances, prothrombin A and prothrombin B, has been accepted by many workers, although it has been violently negated by others, who believe that prothrombin A is merely plasma fibrinogen (E C Loomis and W H Seegers. *Am J Physiol* 148: 563-567, 1947). The present authors, working on Quick's hypothesis, found that hepatectomy in the dog was followed by a reduction in both the A and B components, and that an excess of either could partially compensate for a deficiency in the other.

They therefore studied mixtures of plasmas artificially so modified as to contain large amounts of either prothrombin A or B, respectively. In such mixtures, where from 0 to 100 per cent of the A plasma was added to 100 per cent to 0 per cent of the B plasma respectively, they found that an increase in the prothrombin time appeared only when the concentration of prothrombin A was over 5 per cent and that of prothrombin B under 50 per cent, or when prothrombin B was over 70 per cent and A less than 10 per cent. In other combinations, the two plasmas were mutually complementary. The conclusion was reached that a given clinical prothrombin time might be the result of varying degrees of deficiency of each complex, but that the exact nature of the abnormality might remain unknown.

These results are open, as the authors realize, to the criticism that the available plasmas must all be



mixtures of the A and B components, since it is virtually impossible to prepare completely purified substances, and that plasma from several species—dog, rabbit, human—were mixed. They are open to the greater criticism that the Quick hypothesis of two such separate substances is probably incorrect, and that results based on such hypothesis must be explained on other bases. The unitary nature of prothrombin seems in the stage of becoming well established.

S E

**THROMBOPLASTIC ACTIVITY OF THE URINE** *L. M. Tocantins and J. N. Lindquist* From the Division of Hematology, Department of Medicine, Jefferson Medical College and Hospital, Philadelphia, Pa. *Proc Soc Exper Biol & Med* 65: 44-49, 1947

Experimental data are reported indicating that intact, protein-free and cell-free urine, as well as dialyzed urine, possess clot promoting activity when tested with normal and hemophilic blood, the behavior of the active agent resembling that of thromboplastin. Hemophilic urine frequently exerts as potent an effect as normal urine, occasionally, indeed, the former possesses greater clot accelerating properties than urine from normal individuals. Thus, an explanation is afforded for the frequent occurrence of clot formation in the renal pelvis of hemophiliacs with hematuria, the clotting time of whose blood, aspirated or shed from vessels elsewhere, may be markedly prolonged.

C P E

**THE ACTIVE PRINCIPLE OF PLACENTAL TOXIN THROMBOPLASTIN, ITS INACTIVATOR IN BLOOD ANTITHROMBOPLASTIN** *C. L. Schneider* From the Dept. of Physiology, Wayne University College of Medicine, Detroit, Michigan. *Am J Physiol* 149: 123-129, 1947

It has long been known that extracts of human placenta are toxic when injected into animals. The amount of toxic substance in a placenta can be determined quantitatively by a mouse-assay technic, as well as the amount of a corresponding inactivating material present in human blood. The nature of the placental toxin was tested by the author, and its chemical and physiological characteristics compared with those of thromboplastin. Placental toxin and thromboplastin behaved similarly in several respects. When serial dilutions of thromboplastin and of placental extract were made and tested, it was found that both showed similar curves of inactivation respectively by heat, by acidity, and by human serum. In addition, both behaved similarly in the ultracentrifuge. Heparin (antithromboplastin) was found to protect against the placental toxin in mice, both *in vitro* and *in vivo*.

Schneider concludes that the placenta is a potent source of thromboplastin, and that the toxic substance obtainable by extraction of placenta or of the pregestational uterus is thromboplastin. He speculates further whether this toxin might be related to the toxemias of pregnancy, and finds:

1. The anatomic lesions of eclampsia are consistent with thromboplastin poisoning. Mice given placental toxin showed clotting of blood in their small venules.
2. The concentration of anti-thromboplastin was found to be increased during pregnancy.
3. Pregnant animals are more sensitive to thromboplastin than are nonpregnant animals.
4. There are two reports in the literature suggesting that the anti-thromboplastic substance in the serum is decreased in eclampsia.

Therefore, the author speculates, Cautious administration of heparin might be indicated in pre-eclampsia.

The concept is an interesting one. The nature of the toxemias of pregnancy is still controversial, however, the postulate that some sort of toxic substance is the cause has long been held. Termination of toxemia by termination of pregnancy supports the thesis that the 'toxin' arises from the products of conception. Whether thromboplastin could be the causative agent remains to be demonstrated. It is difficult to see how it could explain all the findings of eclampsia, such as persistent hypertension. The present highly preliminary report suggests, however, certain questions. Thromboplastin occurs in the normal as well as the eclamptic placenta, what is the factor which allows a normal pregnancy in the one individual, and toxemia in the other? Can eclampsia-like lesions be produced experimentally in animals by means of thromboplastin? Is the blood of eclamptic individuals high in thromboplastin content?

S E

**DONNEES RECENTES SUR LA COAGULATION** (Recent statements on coagulation) *P. Frélicherq* (Actualités biochimiques M. Florkin) Desoer Edit. Liege, Belgique 1946

The chief interest of this important monograph resides in the fact that it is a synthesis of the Anglo-Saxon and Continental conceptions on coagulation. The first stage of the coagulation is the main object

of this study Frédéricq stresses the necessity of opposing the tissue extracts (lipoproteins) to the cephalin (lipoid). They differ on three main points (1) Tissue extracts act in the same way on glass and paraffined glass, whereas lipoids are nonactive in the presence of paraffin, (2) Intravenous injection of lipoids is harmless, while tissue extracts provoke intravascular clotting, (3) Coagulation of hemophilic plasma is strongly accelerated by tissue extracts, but hardly affected by lipoids. According to Frédéricq, this discrepancy is explained by the necessity for the lipoids to react with a plasma factor. This factor quickly disappears in stored blood, and is named by Frédéricq 'Trypsin', it is a thermolabile euglobulin, probably identical to the plasma factors described by Widenbauer, Reichel, Leggenhager, Lozner and Taylor, Feissly, etc., and is lacking in hemophilia. It is through this trypsin that the calcium-platelet system, which is not in itself proteolytic, is able to activate the prothrombin. According to Frédéricq, the Quick's time does not only refer to the prothrombin level, but also to the trypsin concentration. To him, the A and B components described by Quick for the prothrombin, are the trypsin (A) and the prothrombin itself (B). The Russell venom, in opposition, is concerned only with prothrombin, being itself proteolytic. This thorough study includes 303 bibliographic references.

J P S

A CASE OF PURPURA FULMINANS WITH FIBRINOGENOPENIA IN ASSOCIATION WITH SCARLATINA *H Daggve*

From the Blegdamshospitalet, Copenhagen, Denmark. *Acta Med Scand* 127: 382-395, 1947

The author discusses the history of purpura fulminans first described by Henoch in 1887 and points out that one third to one half of the cases occur after scarlet fever. A number of cases, however, showed no preceding disease. In scarlet fever the purpura generally appears in the second to fourth week of the primary disease, which may be strikingly mild. Hematologic data are usually normal, except for secondary anemia after the bleedings. Blood cultures almost always are negative. The author found a prolonged coagulation time and this has also been noted by another observer.

The case was a boy of 3½ years. Scarlet fever was not severe. On the twenty-first day after the beginning of the disease large blood spots appeared after a hot bath. Both legs were swollen and later there was swelling and bluish discoloration of the lumbar region. A coagulation time of 14 minutes, with a small, loose clot, later increasing to 30 minutes was the most remarkable hematologic finding. The patient ran a high fever and the hematomata increased. He was treated with two large blood transfusions and vitamin K as the prothrombin time was increased. Determinations of fibrinogen were then performed and the very low value of 0.06 grams per cent was noted. Probably the fibrinogen content was still lower before the transfusions. After two further transfusions the condition improved in spite of several abscesses which formed in the hematomata. The antistreptolysin titer increased from 160 to 900. There was continued improvement and an examination nine months later was normal.

This is obviously an instance of the very rare condition, fibrinogenopenia. It should be noted that the patient was first regarded as suffering from hypoprothrombinemia as the Quick test was positive. The poor quality of the clot however led to the correct diagnosis.

Fibrinogenopenia has been noted in 2 cases of purpura fulminans. In one case following chicken pox the patient recovered after large transfusions. The blood remained fluid for two days and the plasma fibrinogen was 0.015. The other case died 24 hours after the bleeding was noted, and there was no previous malady.

Penicillin against the streptococcal infection and large blood transfusions combined with convalescent serum from scarlet fever patients is obviously the treatment of choice.

Another instance of purpura fulminans was lately published by S. Heinild, *Acta Paediatrica*, 34: 147, 1947.

In this instance there was no previous malady noted in a 3 weeks old child which died in 46 hours from the onset of the disease. Hemolytic streptococci were cultured post mortem from the hematomata, the liver and the ascitic fluid. Penicillin and blood transfusions were not given, nor was plasma fibrinogen determined.

J W

PURPURA FULMINANS AND ITS RELATION TO SCARLATINA *H Frödin* From the Stockholm Hospital for Infectious Diseases, Stockholm, Sweden. *Acta Paediatrica* 34: 217-233, 1947

The case is that of a boy 5 years old, who, on the fifty-seventh day after scarlet fever developed typical erythema exudativum multiforme with slight temperature. On the sixty-third day ecchymoses spread over both legs, and small petechiae were noted on the trunk. Hematuria developed. Penicillin and blood

transfusions were given. There were maximally prolonged bleeding, coagulation and prothrombin times, but fibrinogen was not determined. Blood culture was negative. Exitus after three days of purpura. The very long coagulation time seems to indicate that a lack in fibrinogen may have been the cause of the bleeding and makes the importance of large blood transfusions obvious.

A survey of the cases with purpura from the Hospital for Infectious Diseases in Stockholm during the last 25 years showed no other instances of purpura fulminans.

J W

#### PURPURA NECROTICA. A POSSIBLE CLINICAL APPLICATION OF THE SHWARTZMAN PHENOMENON. J H Sheldon

From the Royal Hospital, Wolverhampton, and the Hallam Hospital, West Bromwich, England. Arch Dis Childhnd 2: 7-13, 1947

The differential diagnosis of purpuric lesions is often very difficult, especially in children, and if the purpura cannot be readily explained as a symptom of one of the more commonly recognized diseases (leukemia, idiopathic thrombocytopenia, hemophilia) there is a tendency to list it as one of the heterogeneous group known as vascular purpura. From this wastebasket, from time to time, specific subtypes of vascular purpura are collected and reported as entities. Among such more or less definite disorders are pseudo-hemophilia, Schönlein-Henoch purpura, and purpura simplex. In the present report, Sheldon separates an additional small group of purpuric disease to which he gives the name purpura necrotica.

Three cases are described in girls aged 11 months, 3 years, and 5 years respectively. In each the history was essentially the same: there was a sudden onset of pains in the limbs, sometimes with swelling of the eyelids, hands, feet, knees, and ankles, then easy bruising and severe purpuric eruptions best described as hemorrhagic bullae. The notable feature of these purpurae was their geometric contours: they formed triangles, squares, and linear structures. After two weeks of acute illness, recovery began. During this stage, the lesions became stony hard, then the black overlying skin peeled off to leave healthy granulation tissue, and finally healing occurred by the slow granulation process over a period of from six weeks to two years. The large lesions left tremendous scars which resembled, because of their depth and extent, healed gunshot wounds. The children had no other residua. Little detailed laboratory study was possible. The platelets were normal in the 2 cases in which they were enumerated. Slight secondary anemia and leukocytosis were present. The bleeding and coagulation times were normal in one case. Cultures of the lesions gave no growth.

Two other similar cases were gathered from the literature by Sheldon, in one of whom splenectomy was followed by death because of sepsis. Sheldon speculates upon the nature of the disorder, and especially upon the curious configuration of the lesions. He notes that several of the angulated lesions occurred in areas of pressure: for example, one large buttock lesion corresponded to the pressure exerted by a pillow upon which the patient had sat for some time, and other smaller lesions were consistent with pressure from wrinkled bedclothes. He points out that, if somehow the area had become sensitized, subsequent prolonged pressure over a period of time might result in severe local tissue damage with accompanying hemorrhage (purpura). In the experimental Schwartzman phenomenon, an intradermal injection is used to sensitize a local area which becomes engorged and necrotic after a subsequent systemic (intravenous) injection. In the present disease, according to Sheldon, it is possible that the angioneurotic edema and periarticular swelling seen early in the course may sensitize the tissue cells which, upon pressure by bedclothes, chairs, etc., break down in geometric patterns with the resulting curious picture of purpura. This concept, of course, does not explain the fundamental nature of the disorder, which Sheldon believes is probably an allergic purpura, the subsequent peculiarities being the result of the Schwartzman-like phenomena.

S E

#### CONGENITAL THROMBOCYTOPENIC PURPURA. J Talmadge and B Berman. From the Pediatric Service, St Louis City Hospital, St Louis, Missouri. J Pediat 30: 691-695, 1947

Thrombocytopenic purpura, developing in the neonatal period, is reported in three successive offspring of a mother with idiopathic thrombocytopenic purpura treated with complete relief by splenectomy three years prior to the first pregnancy. No observations are recorded pertaining to the course of her hemorrhagic diathesis following splenectomy, apart from prenatal examinations when she constantly exhibited

marked thrombopenia, prolongation of the bleeding time and, during the latter months of the first and third pregnancies, the recurrence of spontaneous bleeding. The three infants exhibited at birth a profound thrombocytopenia and associated manifestations of the disease, spontaneous recovery proceeding in each instance from the third to sixth week post partum and becoming complete within two months

C P E

THE PERIPHERAL VASCULAR SYSTEM AND ITS REACTIONS IN SCURVY. AN EXPERIMENTAL STUDY. R. E. Lee and N. Z. Lee. From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York. *Am J Physiol* 149: 465-475, 1947

Microscopic observations were made under relatively physiologic conditions of the mesenteric capillary bed in normal and scorbutic guinea pigs. The size of the capillaries, the nature of the capillary wall, the status of capillary blood flow, the occurrence of petechiae, and the effects of trauma and of adrenalin were studied.

Scurvy was found to be associated with certain definite abnormalities of the capillaries, especially a dilated peripheral vascular system and a slowing of blood flow. The small arterioles and the precapillary sphincters were found to be unresponsive to adrenalin. Atony and sluggish flow of the venules were marked. No petechiae were present in either the normal or the scorbutic animals, but slight traumata frequently produced petechiae in the guinea pigs with scurvy, but rarely in normals.

Scurvy, therefore, resulted in a decreased responsiveness of the contractile elements of the peripheral blood system, and in dilatation and engorgement of the small venules. The means by which the vascular hypotonia is produced is discussed by the authors, who speculate on the possible implication of the adrenal glands (it is known that the ascorbic acid content of the adrenal glands is low in scurvy, and that a reduction in ascorbic acid results in a depression of the activity of the adrenal cortex). The vascular atony, by allowing sluggishness of blood flow and therefore engorgement, may allow the vascular hemorrhages which are characteristic of scurvy.

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# NEWS AND VIEWS

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The following has been received from the William Buchanan Blood Center

To the Editor

So that you will have some background of the Blood Bank Institute held in Dallas, November 17, 18, and 19, and the formation of the American Association of Blood Banks, I am giving you a few facts in which I thought you would be interested. I am attaching hereto a copy of a Blood Bank Survey\* which I compiled from information contained in questionnaires sent to more than one hundred blood banks throughout the U S A in 1945. Being new in the blood bank field myself when I came to this blood center, I endeavored to procure information concerning the operation of other blood banks and was surprised to find that no one seemed to be in a position to furnish me with such information, therefore I set about trying to procure first a list of blood banks. This was indeed a tedious and drawnout task. After compiling a rather complete list, I then prepared a questionnaire which I submitted to these blood banks, as a result of which the attached survey was compiled, said survey being sent to those blood banks which had furnished me information. You will note that Question 37 asked, "Would you be interested in becoming a member of a National Association of Blood Banks designed to meet the needs and study problems of blood banks in general?" As a result of the interest indicated in reply to this question the Blood Bank Institute was planned and the resulting National Association formed.

At the Institute there were 71 cities, 27 states and 57 blood banks represented. Hawaii, Canada and Mexico also had representatives at the meeting. I am enclosing a copy of the program† as well as the constitution of the American Association of Blood Banks and copies of the five resolutions adopted by the Association, as well as a list of the officers. If you need any further information, please do not hesitate to call upon me. I personally appreciate your interest in this meeting and the resulting Association as it is very dear to my heart, and I am hopeful that much benefit will be derived by all blood banks participating from the Association formed.

Sincerely yours,  
WM BUCHANAN BLOOD CENTER  
Marjorie Saunders, LL B,  
Administrative Assistant

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PROGRAM, NOV 17-19, 1947

## BLOOD BANK INSTITUTE

Need and Possible Scope of an Association of Blood Banks, Dr John Scudder

Blood Banks and Disasters, Dr William C Levin

Blood Program of Hawaii, Dr Leon E Mermod, Mrs Hazel H Bond

*Round Table* Scientific Problems in Blood Banking, Dr E E Muirhead, *Chairman*

Technical and Social Aspects of a Community Blood Bank, Dr J Richard Czajkowski

The Blood Bank in a Charity or City-County Hospital, Dr O R Brines

Nomenclature of Blood Antigens Especially CDE cde (Rh Hr) Group, Dr Joseph M Hill

Origin and Growth of the Belle Bonfils Memorial Blood Bank, Marion R Rymer, Ph D

*Round Table* Discussion of Present and Future Needs for Blood Banks in U S, Dr Sol Haberman

*Chairman*

The Blood Bank Service of the New York Post-Graduate Medical School and Hospital, Dr Lester J Unger

Blood Bank Administration, Dr J W Davenport, Jr

Financial Problems and Procedures, W Quinn Jordan

Survey of Techniques for Blood Antigens and Anti-body Determinations, Dr Sol Haberman

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\* Due to limitation of space, the survey cannot be published *Editor*

† See below

Blood Typing and the Use of Universal Donors, Dr G A Matson  
Statewide Blood Bank Service, ' John Elliott, ScD

Cause and Management of Incompatible Transfusion Reactions and Allied Conditions, Dr E E Muirhead

A Plan for Training Blood Bank Personnel, ' Dr Sol Haberman

*Round Table* Administrative Problems in Blood Banking, ' Dr Joseph M Hill, *Chairman*

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To the Editor

I am sure you know by now that our Blood Bank Institute (held in Dallas, Texas, November 17-19, 1947) resulted in the formation of the American Association of Blood Banks. Although the original purpose of the Blood Bank Institute was for the presentation of methods of establishing and operating blood banks, and for the teaching of new technics for processing blood for transfusions, it soon became evident that those registering for the Institute were also interested in the formation of an association of blood banks.

The newly formed association decided that its membership would be limited to ethical, independently operating and policy making, nonprofit institutions, including those operated by approved hospitals, engaged in blood banking.

The stated purposes of this association are

- 1 To promote and foster the exchange of ideas and materials and the dissemination of information relating to Blood Banking and its technical methodology by education, publicity and research,
- 2 To foster and plan for cooperation in times of disaster,
- 3 To function as a clearing house on questions relating to the training of personnel common to such institutions,
- 4 To keep currently aware of and encourage high standards of service,
- 5 And to promote and foster and aid and abet the extension of similar services throughout the United States and its territories.

The resolutions adopted by the Association were definitely intended to put blood bank problems on a local level with the medical profession by way of the county societies acting in an advisory capacity. Through this approach each organized blood bank could serve the medical profession and the community on the basis of local conditions, demands and patient needs.

The capacity for meeting catastrophies by independent blood banks has been demonstrated in the recent past. During the Texas City disaster the Galveston and Dallas blood banks proved that immediate cooperation of a high order is possible on the part of the independent blood bank even though no previous plan had been formulated. During this disaster the Buchanan Blood Center became the collection and processing center and shipped the blood by air to the John Sealy Blood Bank, which then served as the giving center. In the meantime, the Harris Memorial Blood Bank of Fort Worth was standing by in case the blood demands exceeded our capacities. You might be interested to know that twelve hours from the time of the first explosion (four hours after we had been notified) our blood bank was collecting 100 pints of blood per hour for Galveston. In the future the Blood Bank Association hopes to make such cooperation nation-wide, and even more effective.

Sincerely yours,  
Sol Haberman, Ph D

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# FIRST REPORT OF THE COMMITTEE FOR CLARIFICATION OF THE NOMENCLATURE OF CELLS AND DISEASES OF THE BLOOD AND BLOOD-FORMING ORGANS

To the Editor

A committee for clarification of the nomenclature of cells and diseases of the blood and blood-forming organs held its first meeting in Chicago at the Drake Hotel and Northwestern University Medical School October 25-26, 1947. The committee was sponsored by the American Society of Clinical Pathologists and the American Medical Association.

The function of the committee is to define areas where agreement can be reached, not to determine questions of fact requiring new experimental evidence for conclusive proof

The need for clarification and definition of terms is so urgent, not only for the sake of those now using them but particularly for the sake of the medical students and technicians of the future, that an earnest effort to find and secure general acceptance of a single term, where several with similar definitions are now in use, is thoroughly worth while

The choice of a preferred term, it was agreed, should be based not on historical priority or common usage, but the simplest, clearest and most descriptive term should be chosen. Eponyms and new terms should be avoided wherever possible, without sacrifice of clarity. A real effort should be made to attain consistency between related terms

All recommendations of the committee are to be tentative until they have been widely publicized and acted on by all interested groups. The committee will welcome any suggestions and criticisms. A committee should re-examine the terms every five or ten years. A full report on the Committee's deliberations will be published in a subsequent issue of the Journal

EDWIN E. OSGOOD, M.D.  
Portland, Oregon

#### THALASSEMIA, COOLEY'S ANEMIA OR MEDITERRANEAN ANEMIA?

To the Editor

Since Cooley, in 1925, separated the now well known syndrome as a new entity, various terms have been proposed for its description. Most of these terms, beginning with Cooley's own "Erythroblastic Anemia," are unfortunate misnomers. In 1936, Whipple and Bradford, apparently with more zeal and less deliberation, proposed the name "Thalassemia," which unfortunately is appearing with increased frequency in the literature, due more to the prominence of the authors, rather than to its medical or literary merits.

The Greek word "thalassa" means "sea," just "sea," not the "Great Sea" or even "The Sea." The Mediterranean Sea is called "Mesogeios Thalassa," "mesogeios" meaning "mediterranean," ("amidst the land"). If the Greeks usually meant the Mediterranean, whenever they mentioned the sea, it is just because the Mediterranean surrounds all Greek lands, just as the people of New York refer to the Atlantic whenever they mention the ocean, while the people of San Francisco mean the Pacific.

However the term "thalassemia" is unfortunate from another point of view. According to usage, the first component part of a composite word qualifies the second. Thus, glycemia means sugar in the blood, lipemia, fat in the blood (Vid cholesteremia, azotemia, etc). Likewise, hematuria blood in the urine and not urine in the blood. Hence "thalassemia" should mean "sea in the blood," which, of course, is absurd. Even substituting the suffix "-anemia" instead of "(h)-emia," forming thus the word "thalassanemia," would not convey the intended meaning,

but according to grammatical and etymological rules, should mean "anemic sea," or at best "anemia caused by the sea" (Vid Thalassophobia fear of the sea), or, a condition where the sea is in the blood of the anemic person

I believe that the term 'Mediterranean Anemia,' good or bad, is more adequate, if we care to put geographic limitations to the syndrome, even though we no longer think that the condition has such strict geographic limitations. And, despite all objections to the use of authors' names in medical terminology, 'Cooley's Anemia' may be retained, since it is now precisely understood by all

SAVAS NITTIS, M D

Asst Clinical Professor of Medicine, New York Medical College

Dr Nittis has, I believe, a very good point. Although I have used the designations "target cell anemia" and "Mediterranean target-oval cell syndromes" in writing, I find in conversations that the relatively simple designation "Mediterranean anemia" seems to be increasing in usage. It is certainly indicative of the locale of inheritance of the great majority of cases and although it does not describe the type of anemia, it is far more descriptive than the eponymic "Cooley's Anemia." I, myself, would be inclined to drop the designation of target-oval cell syndromes for Mediterranean anemia. *Editor*

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#### THE "COAGULATIONISTS" A NOTE ON THE FORTHCOMING MEETING

Following the wishes of "coagulationists" expressed at the Chicago meetings last year, we shall this year again plan a special section to bring together papers in this field at one of the sections of the Pathological Society where past experience has shown that we can have a most successful get-together. A luncheon will be held in conjunction with the Federated Societies at Atlantic City, between March 16-19

John H. Ferguson  
Department of Physiology, School of Medicine  
University of North Carolina  
Chapel Hill, N. C.



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# BOOK REVIEW

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*Heparin in the Treatment of Thrombosis*, ed. 2 By J. ERIK JORPES Oxford University Press, London, England, 1946

It was in 1916, while Jay McLarn in Howell's laboratory was engaged in the purification of cephalin obtained from heart muscle, that he found a preparation which, instead of accelerating the coagulation of blood, greatly delayed it. From the first Howell felt that this newly discovered substance would find a suitable application in experimental physiology and possibly in the treatment of disorders of coagulation. A slow appreciation of the need for this type of therapy, and difficulties in the purification of the anticoagulant, delayed adequate fulfillment of this prophecy until 1935, when Best and a group of Toronto workers (Charles, Scott, Murray, Waters, Jaques) succeeded in purifying the substance and demonstrating its thrombosis-preventing properties, experimentally and clinically.

By 1939 enough facts had accumulated to lead Dr. Jorpes to publish a monograph on heparin. The present second edition of this monograph brings together additional facts developed in the past seven years. Chapters I, II and III are concerned with the chemical composition, mode of action and site of formation of heparin. The author's work has been chiefly concerned with these phases of the subject. In narrative style he takes the reader through the steps of chemical identification of the compound and the events which led to tracing the site of formation of heparin to the mast cells of Ehrlich (now designated as 'heparinocytes' by the author).

Chapter IV is devoted to a discussion of methods for the standardization of heparin. The author wisely states that the anticoagulant activity of heparin must be measured in biologic systems, since no physicochemical or color reaction will give reliable results. If, however, as stated, 'The quantitative determination of heparin in its natural milieu is almost an impossible task, how is one to explain the fairly consistent results in titrating heparin (with protamine) when this anticoagulant is added directly to the blood? The fact that neither protamine nor toluidine blue, even in small amounts, can reduce the rate of coagulation of normal blood, must then indicate that heparin as such is not present in the blood, though it may be brought in from the tissues under certain pathologic conditions. This may also account for the fact that no figures are available, and none were found in this book, on the physiological concentrations of heparin in normal human blood. The propriety of referring to heparin as the physiological anticoagulant is, therefore, questionable.'

The remaining three-fourths of the work deals with the clinical applications of heparin, including its use as an anticoagulant for chemical determinations and transfusions. Detailed instructions are given for the administration of the anticoagulant, with a discussion of diseases in which it can be of help. Specific case reports illustrate some of the conclusions. The text is abundantly supported with statistical data, collected principally in the Swedish Clinics. When one pauses to think that the author is a biochemist, it is surprising that the clinical sections are so well handled. Moreover, he manages to maintain a critical attitude, though his enthusiastic espousal of this form of treatment is obvious.

As said by Prof. Learmonth in the foreword, 'The monograph is complete: there is here assembled in a form readily available, an account of every aspect of a subject.' Even a section dealing with dicoumarol and its use in the prevention and treatment of thrombosis is included, presumably because this drug is being used more and more in combination with heparin. Clinicians, physiologists and biochemists may well turn to this book for clear, authoritative information on a drug and a method with increasingly wider applications.

L. M. TOCANTINS

# BLOOD

## *The Journal of Hematology*

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### CLINICAL SIGNIFICANCE OF CARDIAC AND RESPIRATORY ADJUSTMENTS IN CHRONIC ANEMIA

By HERRMAN L. BLUMGART, M D , AND MARK D. ALTSCHULE, M D

#### I INTRODUCTION

THE SUPPLY of oxygen to the tissues depends on the oxygen carrying capacity of the blood and the ability of the cardio-respiratory system to aerate and to transport the blood to each living cell of the body. The purpose of this communication is to review the status of the cardio-respiratory system and the related manifold compensatory mechanisms which provide a maximal supply of oxygen to the tissues in the presence of anemia. An understanding of these adjustments is necessarily based on knowledge of the changes in cardio-respiratory dynamics and on an appreciation of the nature of the clinical manifestations.

Most of the physiologic studies reviewed here have been published during the past twenty years. These two decades were most fruitful, for it was during this period that it became possible to study the same patients before and after effective therapy. This was the direct consequence of the discovery by Minot of the therapeutic effectiveness of liver, and the studies in iron therapy which were also carried out to an important extent in his laboratories.

#### II CHANGES IN CARDIOVASCULAR DYNAMICS

##### *The Minute Volume Output of the Heart*

Numerous investigations have shown that with anemia there is an increased cardiac minute volume output of the heart. The relation between the severity of anemia and the degree of increase of the minute volume output observed in different studies has not, however, been uniform. Similarly, the relation of the changes in minute volume output to other aspects of circulatory dynamics has also varied in different investigations. This is hardly surprising, when one considers the number of variable factors involved.

The method used to measure the minute volume output of the heart in man have been of necessity indirect, and have depended, until recently, on respiratory techniques. The complexity and technical variations of these methods have made quantitative comparison difficult and the number of measurements inevitably meager for statistical study. In many communications, the actual minute volume output values

From the Medical Service and Medical Research Laboratories, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts

have been related directly to the hemoglobin concentration of the blood without regard for the fact that variations in cardiac minute volume output in normal people tend to be proportional to variations of the surface area and of the oxygen consumption. Anemias, moreover, are frequently related to, and indeed may be caused by, diseases which in themselves affect cardiac output because of fever or nutritional disturbances.

Plesch<sup>86</sup> in 1909 observed that the cardiac output increased when there was a decreased hemoglobin concentration of the blood. Utilizing the Fick principle, he found that the increase in cardiac output roughly paralleled the decrease in hemoglobin. Confirmatory results have been reported by other investigators<sup>15, 22, 77, 78, 10, 67, 75, 80, 92, 97, 100-103</sup> who used a variety of methods, but Kininmonth's results<sup>62</sup> are discordant. Several excellent studies in which measurements were made in the same subjects both in the anemic state and after improvement are of particular interest.<sup>10, 80, 92, 101, 103</sup> On the basis of twenty-two observations in 8 individuals, Richards and Strauss<sup>92</sup> concluded that there was a definite, though not entirely constant, tendency for the cardiac output to increase with decreasing hemoglobin concentrations, this general relationship as well as the variation disclosed in the individual instances applied to both the cardiac minute volume output and to the cardiac index, or the ratio between cardiac output and surface area. Results obtained by more accurate methods based on cardiac catheterization have become available recently. In a series of anemic patients studied by Sharpey-Schafer,<sup>97</sup> cardiac output was elevated 50 to 150 per cent over the normal average of 5.3 liters per minute, and generally was greatest at the lowest levels of hemoglobin concentration. Similar results have been reported by others utilizing the same technic.<sup>16</sup>

There has been no uniform agreement as to the level to which the hemoglobin concentration falls before the cardiac output is definitely increased. Dautrebande<sup>88</sup> observed an increase only when the hemoglobin fell below 50 per cent. Although others have expressed the opinion that a considerably lesser degree of anemia may cause a measurable rise in cardiac output,<sup>75</sup> no unequivocal rise in the cardiac output was observed in the studies by Brannon and his associates<sup>15</sup> until the hemoglobin concentration was less than seven grams per cent.

#### *Relation Between Cardiac Minute Volume Output, Metabolic Rate, and Coefficient of Oxygen Utilization*

When the oxygen carrying capacity of the blood is diminished in a patient with anemia, two mechanisms are available to maintain an adequate supply of oxygen to the tissues, these mechanisms may act singly or together. The first mechanism which may compensate for deficient concentration of hemoglobin consists of an increased delivery of blood to the tissues consequent to increased cardiac output. If the concentration of hemoglobin were 50 per cent of normal and the cardiac minute volume output and blood flow were doubled, the amount of oxygen withdrawn from each cubic centimeter of blood in the capillaries might be one-half the normal, but the total amount of oxygen given off to the tissues would be unchanged. Despite the low arterial blood oxygen content, the venous blood oxygen

concentration would remain normal. Since the venous blood oxygen tension reflects that of the tissues it is clear that tissue anoxia would be prevented. Sole reliance on this mechanism would require the heart to expend a greatly excessive amount of energy in order to prevent tissue anoxia, and the circulatory reserve would be encroached upon to a marked degree.

The minute volume output of the heart, other factors being equal, is also related to the total oxygen consumption of the body. If the total oxygen consumption of the body were markedly increased in the presence of anemia, part of the increased minute volume output of the heart would be attributable to this factor. Most observers, however, have found normal values while others have observed small increases in some instances, this will be discussed below. Thus, the increased output observed in anemia can be ascribed only slightly, if at all, to increased total oxygen consumption of the body.

The second mechanism available for maintenance of an adequate supply of oxygen to the tissues consists of more nearly complete abstraction of oxygen from the blood as it passes through the capillaries. Normally, 100 cc of arterial blood contains approximately 21 cc of oxygen. Under normal basal conditions, only about 5.5 cc, i.e., approximately 30 per cent, are removed from the blood as it passes through the capillaries. The remaining 15.5 cc may be regarded as reserve oxygen which can be called upon during exercise or other unusual states to prevent asphyxia of the tissues. The anemic patient, to the extent to which he relies on the mechanism of more complete oxygen abstraction, diminishes his reserve oxygen and sacrifices this factor of safety; the degree of sacrifice would depend upon the severity of the anemia. Moreover, the gradient between the oxygen tensions of capillary blood and that of the cells of the tissues must be less under these circumstances.

The data available in the literature clearly demonstrate that the burden of anemia on the cardiovascular system is distributed, part being assumed by an increase in the cardiac output per minute and a part by the increased percentage, i.e., coefficient of utilization, of oxygen by the tissues. The actual amount of oxygen abstracted from the blood as it courses through the capillaries of an anemic patient is less than normal, but the ratio between the amount abstracted and the subnormal amount initially present in the arterial blood is actually greater and is reflected in the increased percentage of oxygen utilization uniformly observed in patients with severe anemia. It should be noted that normally an A-V oxygen difference of 5.5 grams per 100 cc of blood signifies an A-V difference of approximately 30 per cent of the available oxygen. If there were no increase in cardiac output in severe anemias with less than 30 per cent hemoglobin, the amount of oxygen which could be delivered to the tissues, even with 100 per cent abstraction of oxygen, would be less than the normal oxygen supply. In the most severe degrees of anemias, 80 to 90 per cent of available oxygen is removed, were it not for the increased percentage abstraction, higher values of cardiac output than those actually observed would be required to maintain adequate minute oxygen supply. Thus, increased cardiac output and increased percentage removal of available oxygen are two adjustments which serve to maintain adequate oxygen supply.

The above considerations bear on the interesting question of what should be considered "the normal cardiac output," a concept which has important theoretic as well as practical clinical implications. The normal cardiac output must be considered the cardiac output which is found in healthy subjects at rest and free of discomfort or emotional tension, at medium ambient temperatures and with normal rates of body oxygen consumption. The normal cardiac output varies considerably but is proportional to the surface area, the cardiac index, i.e., ratio of cardiac output per minute to surface area, being relatively constant in all normal subjects. The cardiac index is proportional to metabolic rate. In the presence of congestive failure due solely to cardiac disease there is a decreased cardiac index in relation to bodily requirements for oxygen which remain unchanged or are even increased.<sup>2</sup> When, however, anemia supervenes in the presence of congestive failure the index is not as low as one would anticipate on the basis of congestive failure alone, the cardiac output being somewhat increased rather than diminished in relation to total body oxygen consumption of normal subjects. For example, in the presence of severe anemia with arterial oxygen content of five volumes per cent the cardiac index is approximately seven.<sup>15</sup> This elevation of the cardiac index to more than twice the normal of healthy subjects is the increase whereby the requirements of the body for blood and oxygen are met. An elevation less than this constitutes circulatory insufficiency even though the output at such times may be larger than that of normal subjects. When the cardiac index is below that indicated for the degree of anemia, even though markedly above the so-called normal of healthy individuals, the circulation may be insufficient in relation to the increased demands of anemia, congestive failure may ensue.

In the presence of lowered oxygen consumption in anemic patients, the expected increased cardiac output may not always be present. Thus Starr et al.<sup>100</sup> state "The two cases of anemia did not show the increased cardiac output which we expected. Both of them appear to have reduced their basal metabolism to a point where a normal cardiac output will carry the necessary oxygen. In one of these patients, starvation may well have been the cause of this decrease. This method of compensating for anemia is not that usually described." Similarly in patients with anemia of myxedema, the cardiac output may be low but adequate for the lowered metabolic requirements, congestive failure does not develop.

This general principle applies equally to other conditions which demand an increased output, such as thyrotoxicosis, arterio-venous aneurisms, pregnancy, febrile states, and, indeed, exercise.

### *The Velocity of Blood Flow*

The relation between volume flow and velocity flow of liquids of fixed viscosity through tubes of known diameter is a simple one and is expressed by the equation,  $V = A/\pi r^2$  where  $V$  is velocity flow expressed in seconds,  $A$  is the volume flow per second and  $r$  is the radius of the tube. If other factors remain equal, an increase in volume flow will be accompanied by a proportional increase in the velocity of blood flow. With the somewhat decreased viscosity of blood in anemia, an additional factor tending to increase the velocity of blood flow is operative. The extent to which

pulmonary blood flow is accelerated in the presence of anemia was studied by Blumgart, Gargill, and Gilligan<sup>11</sup> using the radioactive method. Thirty-two complete series of measurements were made in 29 subjects with pernicious anemia and with anemia secondary to a variety of diseases. The results showed that, while there were considerable variations, the velocity of blood flow through the lungs in these patients generally tended to increase in proportion to the degree of anemia. A linear relationship between the increased cardiac output and accelerated velocity of blood flow was observed by Stewart et al.<sup>103</sup>, the greater the cardiac output, the shorter the circulation time. The increase in velocity observed by the latter investigators was of somewhat greater magnitude than that reported by Tarr, Oppenheimer and Sager,<sup>106</sup> but similar to those results recorded by Blumgart et al. With increased speed of blood flow through the lungs, accelerated pulse rates were observed. Other studies of circulation time in patients with anemia have been reported by many authors<sup>4, 9, 10, 13, 33, 36, 38, 56, 60, 63, 66, 70, 71, 74, 84, 106, 112</sup>, all are in accord with the above.

### *Regulation of Peripheral Blood Flow*

Calculation of the average peripheral resistance throughout the body in patients with anemia indicates that a definite decrease is present. However, the state of the small blood vessels is not uniform everywhere. Thus plethysmographic measurements of blood flow in the arms<sup>1</sup> and studies based on estimation of local arterio-venous blood oxygen or carbon dioxide difference in the arms<sup>3, 25, 30, 43, 51, 76, 79, 86, 92</sup> show accelerated flow while blood flow in the hands is diminished<sup>1, 104</sup> in anemic patients. Direct observations of the capillaries in the skin of the fingernail fold show marked vasoconstriction<sup>30, 50</sup> and flow is slow.<sup>30</sup> The occurrence of increased peripheral flow everywhere except in the hands is identical with the pattern of flow seen in simple anoxia such as is produced in normal subjects breathing air deficient in oxygen.

Measurements of antecubital and mixed venous blood oxygen content yield very low values.<sup>3, 15, 25, 51, 76, 79, 86</sup> In spite of the fact that the tissues are given enough oxygen for their basal requirements, as shown by the fact that total oxygen consumption is not lowered in anemia, the low tissue oxygen tensions which exist result in a diminution of the margin of safety. The occurrence of intermittent claudication of the calves in patients with anemia has been emphasized by Pickering and Wayne.<sup>85</sup> In accord with the concept that the tissues are anoxic is the fact that blood lactic acid values are often elevated in patients with anemia<sup>58</sup> and the ability of the body to metabolize intravenously injected lactate is impaired.<sup>21, 45</sup> After exercise the oxygen debt is greatly increased in anemic patients<sup>81</sup> presumably as a consequence of the accumulation of excessively large amounts of lactic acid.

### *Visceral Blood Flow*

<sup>1</sup> *Kidneys* The observations of Bradley and Bradley<sup>14</sup> on renal blood flow in anemia are of interest in that they afford data indicating the presence of selective changes in vasomotor activity. The renal blood flow is greatly diminished in patients with chronic anemia, evidently as a consequence of localized vasocon-

striction. Calculations of the filtration fraction suggest that afferent vasoconstriction is somewhat more marked than that in the efferent arterioles of the glomeruli. Although flow of blood through the kidneys is reduced by a third or a half, the amount of *plasma* presented for filtration to the glomeruli per unit of time as a rule is almost normal because of the low hematocrit values which occur in anemia. Accordingly nitrogen retention is uncommon. On the other hand the observed reduction in blood flow may be related to salt retention observed by Strauss and Fox<sup>105</sup> in patients with anemia (see below). Evidence of impaired tubular function, presumably consequent to anoxia, is also presented by the Bradleys.<sup>14</sup>

2. *Bram* Himwich and Fazekas<sup>13</sup> recorded observations on arterial and jugular venous blood gas concentrations in one anemic patient, which indicate that the flow of blood through the brain is abnormally rapid in anemia, the venous blood oxygen concentration, however, and presumably the tissue oxygen tension in the brain, is very low. These authors ascribed the development of mental symptoms in their patient to anoxia.

### *The Metabolic Rate in Anemia*

A review of the available data indicates there are no striking deviations from the normal total oxygen consumption of the body as a consequence of anemia *per se*. The problem is complicated by the fact that the metabolism of the body may be influenced considerably by the disease which causes the anemia.<sup>26</sup> In pernicious anemia, Boothby and Sandiford<sup>12</sup> observed that approximately 10 per cent of their patients had a metabolic rate above plus 20 per cent. Similarly, an average increase in basal metabolic rate of plus 20 per cent was observed in the 5 patients with pernicious anemia studied by Stewart et al.,<sup>107</sup> a decrease to an average of plus 6 per cent occurring during remission induced by treatment. In patients with iron deficiency anemia, the metabolic rate is increased less frequently and is often normal or below normal. In the 18 patients studied by Brannon et al.<sup>15</sup> the average metabolic rate in patients with less than 7 grams per cent of hemoglobin was plus 13, as compared with plus 5 per cent in patients with hemoglobin between 7 and 13 grams, and minus 7 per cent in their normal subjects. In general, the rise in oxygen consumption by the body in anemia is at most small or frequently absent, even when present, the increase in cardiac output and velocity of blood flow can be attributed only in small part to this rise in oxygen consumption.

### *Blood Volume*

Neither the carbon monoxide method nor the dye methods measure absolute blood volume in normal subjects, experience with pathologic subjects tends to confirm and extend this conclusion. Either method may on occasion give the larger value and it is not possible to assert that either one consistently measures absolute blood volume, both methods apparently tend to exaggerate the true blood volume. The dye method also generally gives excessive values for the plasma volume.<sup>63</sup> It is fairly clear, however, that most methods give fairly reliable qualitative but not quantitative measures of the relative plasma volume. Observations by various investigators of the blood volume in anemia reveal that the blood volume is some-

what reduced<sup>11, 30, 34, 35, 38, 97</sup>, the most thorough studies are those of Gibson et al.<sup>34, 35</sup> The mean plasma volume per kilogram of body weight is usually within the limits of normal, however, so that the diminution is a reflection of reduction in total circulating red cell mass.

### III. CHANGES IN RESPIRATION

#### *Alterations in Ventilation*

The respiratory minute volume is often increased in anemia, even beyond what might be expected in the presence of occasional elevations of the metabolic rate.<sup>8, 16, 57, 64, 66</sup> Some authors have found no striking change in certain instances.<sup>23, 92</sup> Increases in rate and in depth of respiration participate in the elevated respiratory volume. Although these findings suggest the effects of simple anoxia, it is clear that other influences operate in patients with anemia.

All authors who have measured the vital capacity in patients with anemia have found lowered values in many of their subjects.<sup>11, 57, 64, 87</sup> More detailed studies, involving estimation of the subdivisions of the vital capacity, i.e., the reserve and complementary air volumes, show these to be lowered likewise.<sup>64, 87</sup> The residual air is increased somewhat.<sup>64, 87</sup> These deviations from the normal are similar to those observed in pulmonary congestion or edema and denote a loss of elasticity and expandability. Changes of this type favor the occurrence of exertional dyspnea.

#### *Blood Gases and Their Relation to Dyspnea*

Studies of the blood gases in anemia are of importance in understanding the dyspnea which may occur in this disorder. Arterial anoxia obviously must exist when the hemoglobin level is reduced. Although some of the earlier observers reported also that low arterial blood oxygen saturation was common,<sup>55, 86</sup> most workers agree that the saturation is normal in patients at rest.<sup>43, 49, 51, 79</sup> Accordingly it is apparent that the degree of pulmonary congestion and/or edema present in severe anemia is not sufficient to interfere with maximal oxygenation of the blood. On the other hand, during exertion, arterial blood oxygen saturation falls markedly,<sup>49</sup> indicating inefficiency of the respiratory mechanisms under conditions of strain. The reduced oxygen saturation of the arterial blood conceivably might be due to some qualitative alteration of the hemoglobin in anemia, however, the oxygen dissociation curve of blood from anemic patients is normal at all pH levels found in man.<sup>25, 91, 99</sup>

As pointed out above, the tissue oxygen tensions or at least the gradient between blood and tissues must be greatly lowered. This phenomenon exists in the brain, as was shown by Himwich and Fazekas<sup>48</sup> and provides an additional mechanism for hyperventilation.

Another group of factors important in consideration of the respiratory dynamics of anemia is related to peculiarities of carbon dioxide transport in that disorder. Joffe and Poulton<sup>59</sup> showed by means of experiments *in vitro* that erythrocytes are important in carrying carbon dioxide, and Smith, Means and Woodwell<sup>96</sup> further showed that red cells *in vivo* may actually carry most of the carbon dioxide given off by the tissues. Accordingly it is clear that a fall in erythrocyte count must im-



pair transport of that gas. There is an apparent contradiction in this concept in the fact that the whole blood of anemic patients has a carbon dioxide combining power which is in or even above the upper normal range<sup>7 19, 23, 51 51, 79 98</sup>, the carbon dioxide combining power is estimated using whole blood, and since each unit of the blood in anemic patients contains relatively more plasma and fewer cells than normal, the great carrying capacity for carbon dioxide of plasma as compared to cells, which exists normally, results in the small increase observed in the whole blood. However the role of plasma in the transport of carbon dioxide given off by the tissues may be minor, that of the erythrocytes more important, this is evidenced by the fact that plasma and red cells do not participate equally in the changes to be described.<sup>25 12</sup> The loss in anemia of much of the buffering action normally provided by hemoglobin in the red cells and the resultant flattening of the carbon dioxide dissociation curve<sup>7 19 25 44 51 78 82 109</sup> is highly significant: a given amount of carbon dioxide entering the blood causes much greater increases in carbon dioxide tension and hydrogen ion concentration than occurs when the blood hemoglobin concentration is normal. Hyperventilation, which makes the arterial blood somewhat alkalotic in patients with anemia,<sup>7 19 23 25 92</sup> enables the blood as it traverses the tissues to accommodate this tendency toward acidosis, the arterio-venous difference for pH is thereby increased<sup>3 7 43 92</sup> but the pH of the venous blood,<sup>7 29</sup> and presumably of the tissues, remains normal.

It has been noted that some types of chronic anemia are associated with less exertional dyspnea than others of the same degree and chronicity but of different types. Clearly, factors other than those discussed above must be important in this regard. In addition to hemoglobin, the red cells contain other respiratory enzymes, including carbonic anhydrase. The latter catalyzes the reaction  $\text{HCO}_3 \rightleftharpoons \text{CO}_2 + \text{H}_2\text{O}$  in either direction, depending on concentrations of the substrate materials. Earlier work with this enzyme tended to minimize its importance in the etiology of dyspnea because, according to the methods used in the past, there is such an enormous excess of this enzyme that it was considered impossible that a deficiency could ever exist, in the adult at least. There are, however, certain errors in these older methods which led to the finding of falsely high values in normal blood.<sup>72</sup> By the use of a newer method it has been found that the amount of activity of the enzyme in normal blood is so small that a decrease, because of disease, would lead to serious impairment of the rate of absorption of carbon dioxide by the blood in passing through the tissues and the release of carbon dioxide from the blood in passing through the lungs.<sup>72</sup> Several workers<sup>62 67 68, 72</sup> have studied the relation between carbonic anhydrase activity and hemoglobin content of the blood in normal subjects and patients with anemia. It has been found that anemias due to blood loss, malnutrition, chronic infection, uremia or leukemia are associated with a proportional reduction in blood carbonic anhydrase activity which is parallel to the decrease in hemoglobin level.<sup>72</sup> Patients with any of these types of anemias, therefore, not only have decreased oxygen carrying capacity but also may have a deficiency in ability to take up carbon dioxide from the tissues and to release it in the lungs. Therefore, not only is anoxia responsible for their hyperventilation and dyspnea on exertion but carbon dioxide accumulation in the tissues is probably also a factor.

On the other hand, patients with Addisonian pernicious anemia may have no deficiency in blood carbonic anhydrase activity<sup>67, 68, 72</sup> and consequently suffer from no impairment of carbon dioxide transport and excretion from lack of the enzyme itself. The reason for this difference between macrocytic and micro- or normocytic anemias is not clear.

In summary, the following factors, many of which are closely interrelated, are operative in the production of dyspnea in anemic patients: the increased respiratory minute volume, the decreased vital capacity and its subdivisions, abnormalities in carbon dioxide transport and dissociation, reduced arterial oxygen capacity and the decreased blood oxygen saturation during effort, the frequently observed elevated blood lactic acid values.

#### IV CLINICAL MANIFESTATIONS OF CHRONIC ANEMIA

##### *Cardiovascular and Respiratory Symptoms and Signs in Anemia*

With the exception of active rheumatic infection, bacterial endocarditis, periarteritis nodosa, disseminated lupus erythematosus, and advanced organic tricuspid disease, organic cardiovascular disease does not cause anemia. Various cardiovascular symptoms and signs occur, however, in the presence of anemia and have attracted the interest of many observers for more than a century. Practically all investigators have concerned themselves with isolated aspects of the problem. Excellent comprehensive clinical studies of the cardiovascular system in anemia were, however, presented by Ellis and Faulkner in 1939<sup>28</sup> and by Wintrobe<sup>113</sup> and Hunter in 1946,<sup>54</sup> the last named also providing an extensive bibliography, which will therefore not be repeated here. Wintrobe<sup>113</sup> also includes an excellent digest of some of the physiologic adjustments of the cardiovascular system in anemia.

1 *Symptoms* Lassitude, anorexia, dizziness, palpitation and breathlessness on exertion generally are found in patients with moderate or severe degrees of anemia. In even the severer grades of anemia, however, breathlessness is rarely present at rest, and orthopnea and paroxysmal dyspnea are absent. Palpitation likewise is generally experienced only with exertion and implies an increase in the rate as well as in the force of the cardiac impulse. Anginal pain may occur particularly on effort.

2 *Physical signs* Pallor of the skin and mucous membranes, changes in the tongue and finger nails, and slight degrees of edema, particularly over the sacrum and lower legs, are characteristic of the anemic state. Increased vigor of arterial pulsation with a widened pulse pressure was frequently observed by Sharpey-Schafer<sup>97</sup> who also described capillary pulsation in the finger tips. In the severer grades of anemia, "pistol shot" sound over the larger arteries, a positive Duroziez sign, and systolic murmurs on auscultation may be manifest. The possibility that these evidences of vasodilatation may be related not to anemia but to associated fever or beriberi must be borne in mind.

3 *Cardiac enlargement* Bamberger in 1857<sup>6</sup> and Friedreich in 1861<sup>32</sup> commented on the pathologic findings of fatty infiltration and degeneration, together with dilatation and increased weight of the heart in patients with anemia. Ball<sup>1</sup> was the first to demonstrate an increase in heart size by x-ray measurements with return to

normal after recovery, this finding has been confirmed by many observers<sup>28, 41, 51, 89, 107</sup> Twelve of the 34 patients studied by Hunter<sup>51</sup> showed definite cardiac enlargement during anemia, with return to normal size in all but 3 following appropriate treatment. In some patients with slight or doubtful degrees of enlargement a decrease in size following disappearance of the anemia was noted. The reduction in size was always generalized, but in addition any straightening of the left border which had been initially present was replaced by a normal concavity. Cardiac enlargement tends to occur more frequently in patients with particularly low hemoglobin levels and, according to Hunter,<sup>51</sup> there is a decrease in enlargement when the hemoglobin percentage rises to the levels of 60 to 95 per cent, variation being observed in different subjects. Regression in cardiac size usually occurred early, often within two or three weeks of the beginning of treatment and at hemoglobin levels still substantially below normal.

Cardiac hypertrophy, however, is also frequently present.<sup>17, 18, 28, 30, 54, 89, 90</sup> The available data seemingly support the opinion of Porter<sup>89, 90</sup> that primary cardiac dilatation is a physiologic adjustment resulting from the increased cardiac output in the presence of a supply of anemic blood through the coronary arteries to the myocardium. If the anemia is rectified early, dilatation disappears and heart size returns to normal. If, however, prolonged dilatation continues with stretching and injury to the myocardial fibers, cardiac hypertrophy inevitably develops.<sup>94</sup> The degree of cardiac hypertrophy is usually not marked, but an instance is cited by Cabot<sup>17, 18</sup> in which the heart weighed 710 grams in the absence of arterial hypertension and coronary arteriosclerosis, the heart of a patient observed by Porter weighed 630 grams.<sup>89</sup>

In general, it may be said that both dilatation and consequent hypertrophy undoubtedly occur in patients with anemia, but that there is no direct relation between the incidence or degree of enlargement and the severity of the anemia. With the return of the hemoglobin levels toward normal, cardiac size usually returns to within normal limits, although occasional instances of persistent slight enlargement may be witnessed. In addition to dilatation and hypertrophy, the anemic heart muscle undergoes a form of fatty degeneration characterized by yellow streaking ("tigering") clearly visible on the endocardial surface.

4 *Heart sounds* A loud and booming first sound is frequently heard in patients with anemia, but no direct relation to the hemoglobin level or the duration of the anemia is apparent.<sup>54, 89, 90</sup> The prolongation and accentuation of the apical first sound may closely resemble the characteristic first sound in mitral stenosis. The aortic and pulmonic sounds are usually not altered, but in some patients an additional third heart sound is heard at the apex. This latter finding has been corroborated by phonocardiography, and must be considered abnormal since the patients were over 30 years of age.<sup>54</sup> Both the booming first sound and the presence of the third sound were associated with a moderate tachycardia, and disappeared when the pulse rate slowed.

5 *Cardiac murmurs* That a blowing systolic murmur commonly is heard at the apex or over the mitral area in anemic patients is generally appreciated. Occasionally, however, such murmurs are heard over the aortic and pulmonic areas, and at

times they may be rough or even rumbling and may be accompanied by diastolic murmurs. Attention should be called to the fact that in rare instances a blowing diastolic murmur may also be heard along the left border of the sternum characteristic of aortic insufficiency. Most observers have encountered these diastolic murmurs only rarely. While Goldstein and Boas<sup>39</sup> reported an incidence of 10 per cent of these murmurs in 39 cases of anemia, these diastolic murmurs occurred only in association with severe degrees of anemia. The blowing apical systolic murmurs tend to be louder, longer, and less affected by posture and by respiration than the same systolic murmurs encountered in healthy young persons. They are usually not transmitted to the axilla. When, however, they have a rough, rumbling quality and are associated with a booming first sound and the slight or moderate cardiac enlargement seen in the presence of moderate or severe anemia, the differentiation from structural mitral disease may offer difficulty and, indeed, the distinction may be impossible. In such unusual instances, decision may have to be deferred until further observations are made when the anemia is alleviated.<sup>113</sup>

It is of interest, that of the 34 patients studied by Hunter<sup>54</sup> while they were moderately or severely anemic and again after treatment, 30 showed cardiac murmurs, always systolic in time except in one case which showed an early diastolic murmur and another a pre-systolic murmur. Six murmurs were described as faint, 16 as moderate, and 9 as rough. In 29 cases, murmurs were heard at the apex, in 21 at the pulmonary area, and in 6 in the aortic area. Twenty had murmurs at more than one site, i.e., mitral and pulmonary in 14, mitral, pulmonary and aortic in 6. An apical murmur alone was present in 9 cases and a pulmonary murmur in 1. Aortic murmurs were always accompanied by both pulmonary and mitral murmurs. The louder murmurs were heard in two and often three areas, and the softer in single areas, generally the mitral. When multiple murmurs were present, they were loudest at the apex, then in the pulmonary area, and least loud in the aortic area. The murmurs were usually diminished when the patients were in the erect position, especially when the intensity had lessened after treatment. In 9 patients the murmurs, though not accompanied by thrills, were loud enough to raise a suspicion of valvular disease, although their distribution made such a diagnosis unlikely. The two diastolic murmurs, one late or pre-systolic at the apex, and the other early in the fourth left interspace near the sternum, led to diagnoses of mitral stenosis and aortic incompetence respectively, until their disappearance with treatment indicated their hemic origin. The incidence of murmurs was not directly related to the severity of the anemia, for they were often conspicuous when it was slight and absent when it was considerable. The duration of the anemia seemed more important.<sup>54</sup> <sup>113</sup> The patients described by Schwartz and Legere<sup>96</sup> illustrate in striking fashion the difficulties which sometimes arise in the differentiation of cardiovascular changes due to anemia from those consequent to serious organic heart disease.

The wide-spread belief that cardiac enlargement is the cause of hemic murmurs was not confirmed by Hunter,<sup>54</sup> since their association was inconstant, and noticeable murmurs were heard when the heart was normal in size and occasionally absent when there was enlargement. It is probable that acceleration of blood flow is an important cause of the murmurs noted, however, the decreased viscosity of the

blood which accompanies anemia favors the development of eddies and therefore of murmurs, as Wiggers<sup>110</sup> pointed out

As mentioned above, cardiac enlargement usually diminishes rapidly while the murmurs tend to persist over a longer period of time. Treatment usually effects a lessening in the intensity of the murmur and most murmurs disappear or become negligible when the blood findings return to normal. In a few instances, however, such murmurs may persist for several months before disappearing.

6 *Tachycardia* Moderate increase in the cardiac rate is nearly always present with moderate or severe anemia, the rate rising to 90 to 100. The relation between the rise in pulse rate and the degree of anemia is variable. In studies on the velocity of blood flow through the lungs, Blumgart et al.<sup>11</sup> observed that the pulse rate was more closely related to changes in the velocity of the pulmonary circulation than to variations in the degree of anemia. This result is hardly surprising since the cardiac rate and the velocity of blood flow are both characteristics of the general circulatory adjustment and as such are more closely related physiologically to each other than to change in the oxygen carrying capacity of the blood.

7 *Arterial blood pressure* In the presence of moderate or severe anemia, a lowered blood pressure is commonly observed with a subsequent rise as recovery from the anemia occurs.<sup>15 85 101</sup> Although the 5 patients studied by Stewart et al.<sup>101</sup> exhibited a rise in arterial pressures amounting in 3 of their patients to as much as 50 to 80 millimeters and 30 to 46 millimeters of mercury in the systolic and diastolic pressures respectively, other workers found lesser changes. Similar, though not as striking or uniform findings, were reported by Brannon et al.,<sup>16</sup> while Bradley and Bradley<sup>14</sup> found no consistent change. This tendency for the blood pressure to rise with recovery from anemia occasionally counterbalances the decreased work of the heart which also occurs at this time due to lessened volume output and velocity of blood flow during recovery, it may result in the work of the heart being approximately the same in some patients during anemia as after recovery.<sup>101</sup> The rise in arterial pressure which may develop is a reflection of the increase in total peripheral resistance which occurs when the need for visceral vasodilatation is no longer present as the hemoglobin level approaches normal. The somewhat widened pulse pressure sometimes seen in anemia with narrowing of the span after recovery is in accord with this concept.

8 *Venous pressure* The right auricular pressure in patients studied by Sharpey-Schafer<sup>92</sup> was usually a high normal. A slight elevation was noted in one of 5 patients by Stewart et al.<sup>101</sup> while no significant alterations were noted in the other 4 patients. No elevation was found by other authors.<sup>38 105</sup> Sharpey-Schafer<sup>97</sup> believes that the increased output is achieved mainly by the raised venous pressure, a concept which requires further verification.

9 *Electrocardiographic changes* Numerous studies of the electrocardiogram in chronic anemia indicate that, while abnormalities occur in approximately a quarter or more of such patients, they are minor in degree and are not specific for anemia. Prolongation of the QT interval,<sup>107</sup> flattening or inversion of T in one or more leads,<sup>27</sup> low amplitude,<sup>108, 114</sup> transitory prolongation of the P-R interval,<sup>85</sup> depression of the ST segment<sup>28</sup> have been noted. A return to normal of the electrocardio-

gram was sometimes observed after treatment, although this did not always occur even when there was no reason to suspect other forms of heart disease. In 2 of the 25 patients studied electrocardiographically by Hunter,<sup>54</sup> gross abnormalities were apparent, but in spite of successful treatment of the anemia, the electrocardiogram was unchanged on re-examination a year later, both patients were in the fifth decade and the changes may have been consequent to coronary arteriosclerosis or other lesions.

The possibility of vitamin B deficiency, digitalis administration and, in some patients, the effect of the disease responsible for the anemia, are difficult to exclude in some of the reported cases although these factors were evidently ruled out in the studies by Ellis and Faulkner<sup>28</sup> and Hunter.<sup>54</sup>

The electrocardiographic changes frequently apparent in chronic anemia are similar to those observed during acute anoxia. That anoxia is an important factor is further supported by the frequently transitory character of the changes in moderate or severe anemia. In some patients, the electrocardiographic changes may represent the summation of anoxia, coexistent coronary arteriosclerosis, and fatty changes in the myocardium.

It is not surprising that the effects of anoxia are prone to occur in the heart during chronic anemia, for even under normal conditions the abstraction of oxygen from the blood as it flows through the heart is relatively great. Thus, mixed venous blood in the right chambers contains approximately 15 volumes per cent of oxygen and, as stated above, this amount of oxygen represents a reserve factor. On the other hand blood from the coronary sinus obtained in observations in man by catheterization technics contains only approximately two volumes per cent. The abnormal reduction of this reserve factor in the heart, the lowering of blood pressure commonly observed in anemia, and the increased work of the heart attendant to the increased output predispose to anoxia.

#### *Angina Factors and Severe Anemia*

Herrick<sup>46, 47</sup> drew attention to patients with severe anemia who developed angina pectoris, and who experienced relief when the anemia improved, this was confirmed subsequently by many other investigators.<sup>16, 20, 27, 28, 54, 61, 65, 68, 111, 114</sup> It is to be expected that in any large series of patients with anemia an occasional instance of coincidental angina pectoris might be encountered. The fact, however, that angina pectoris first appears in some patients with the development of severe anemia and is alleviated by appropriate treatment of the anemia bespeaks an etiologic interrelationship. Such patients are encountered only occasionally and are almost always in the age group in which coronary arteriosclerosis is more common. Cabot<sup>17</sup> and Elliot,<sup>27</sup> however, have both reported cases in which no evidence of coronary artery disease was found postmortem. In most patients of this type, however, it is probable that the heart is damaged to so slight an extent that it is able to maintain an adequate blood flow provided that the oxygen carrying capacity of the blood is normal. In the presence of anemia, however, the increased amount of work necessary to compensate for this condition cannot be accomplished readily, particularly since the blood supply to the heart is affected in common with

that of the rest of the body. Furthermore, the normally large utilization of available oxygen by the heart predisposes to the induction of ischemic muscle pain in severe anemia.

### *Congestive Failure in Anemia*

The necessity for the maintenance of the cardiac output and cardiac work at an abnormally high level for long periods of time, and the delivery to the myocardium of blood deficient in oxygen together favor the occurrence of myocardial insufficiency. Other factors favoring it are present when the patient with severe anemia also is in the age group in which coronary artery disease and hypertension occur, as is common in pernicious anemia and anemia due to carcinoma. Nephritic anemia usually is also complicated by the presence of hypertension. The coexistence of organic cardiovascular disease, added strain upon the heart due to anemia and anoxia of the myocardium are especially prone to result in congestive heart failure. When this syndrome develops the circulatory dynamics change.<sup>97</sup> The right auricular and venous pressures become elevated, the lungs become more congested, and the cardiac output, although often still elevated in comparison to the normal, becomes lowered relative to the values which ordinarily obtain with a given degree of anemia. The arm-to-tongue circulation time in such instances represents the resultant of two opposing factors, i.e., a tendency toward slowing due to congestive failure on one hand and toward acceleration due to anemia on the other, it often lies in or close to the normal range as a consequence. Patients in whom myocardial insufficiency develops as a consequence of anemia have aggravation of their antecedent exertional dyspnea, as is to be expected from the superimposition of the additional mechanisms favoring dyspnea which occur in chronic congestive failure.<sup>9</sup> Such patients are likely to respond poorly to digitalis unless the anemia is corrected. On the other hand, since congestive heart failure develops in patients with anemia when the heart is less seriously damaged than in those in whom cardiac decompensation is referable to heart disease alone, the ultimate outlook, assuming adequate therapy of the anemia, is better in the former than in the latter.

Dyspnea is not necessarily an indication of congestive failure. Dyspnea may be consequent to anemia per se and as such may be the resultant of the action of a number of factors, each of which has been discussed above. These include the effect of low arterial blood oxygen concentrations on the carotid body, the effect of low tissue oxygen tensions directly on the brain, the effect of low oxygen tension in exaggerating lactic acidosis on effort, and the effects of impaired carbon dioxide transport, the latter requires arterial alkalosis to prevent tissue acidosis. These mechanisms all favor hyperventilation and thereby contribute to dyspnea. In addition the changes in the subdivisions of the lung volume indicative of some degree of congestion and/or edema point to a pulmonary factor in the genesis of the exertional dyspnea seen in severe anemia. Still another factor favoring exertional dyspnea is the high cardiac output at rest which reduces the cardiac reserve available for exercise. None of these mechanisms is altered by the administration of digitalis or other forms of therapy directed at cardiac decompensation, they all respond favorably when the anemia is treated successfully.

As in the case of dyspnea, there is a tendency toward edema formation inherent in anemia even in the absence of congestive failure. Older clinicians are aware of this phenomenon and younger physicians still see it occasionally in spite of the earlier diagnosis and more adequate therapy of anemia which obtain today. Peters and Eisenman<sup>83</sup> in a thorough analysis of the relation of the level of the plasma protein to edema in various diseases noted that patients with anemia developed edema at levels of plasma protein well above those seen in other diseases associated with edema, with the exception of congestive heart failure, these authors were unable to explain this phenomenon. The change in capillary permeability noted as occurring in animal preparations under anoxic conditions<sup>69-95</sup> may have some bearing on the problem. On the other hand, it is to be noted that Strauss and Fox,<sup>105</sup> in a study entitled "Anemia and Water Retention," showed that retarded excretion of sodium, varying in degree with the severity of anemia, was present. A tendency of this sort toward salt retention suggests the involvement of some renal mechanism, as pointed out by Bradley and Bradley<sup>14</sup> in their discussion of the occurrence of markedly reduced renal flow in anemia. Many years ago Rowntree and Fitz<sup>84</sup> caused salt retention in animals by inducing renal vascular stasis. It is clear that edema may occur in anemia even in the absence of congestive heart failure. The edema of anemia, like the dyspnea of anemia disappears when the anemia is cured. When exertional dyspnea, edema and the commonly occurring hepatomegaly of severe anemia occur together, the diagnosis of myocardial insufficiency suggests itself. However, the absence of venous engorgement and more especially of orthopnea contradict that diagnosis. Absence of cyanosis is not helpful, for as Lundsgaard and Van Slyke<sup>77</sup> showed, it is impossible for cyanosis to develop under any circumstances in patients with less than five grams per cent of hemoglobin.

## V. SUMMARY

The cardiac and respiratory adjustments in chronic anemia and their clinical manifestations have been reviewed. When the oxygen carrying capacity of the blood is diminished, an adequate supply of oxygen to the tissues is maintained by an increased cardiac output, an increased velocity of blood flow, and a relatively more complete abstraction of the oxygen from the blood as it passes through the capillaries. With the increased blood flow, the average peripheral resistance is decreased but the state of the small blood vessels is not uniform everywhere, the blood flow in the hands and kidneys, for instance, may be reduced, while that of other parts of the body is increased. The total oxygen consumption of the body in anemia is not strikingly altered. The blood volume generally is slightly reduced but the plasma volume is normal.

The deviations from the normal values vary from patient to patient, but generally are definite when the hemoglobin values are less than 50 per cent and are greatest at the lowest levels of hemoglobin concentration.

The close interrelationship between the cardiovascular and respiratory systems is exemplified by the coincident changes in the respiratory system in anemia. The rate and depth of respiration often are increased together with a lowering in the vital capacity and its subdivisions, the reserve and complemental air volumes. The resid-



ual air is somewhat increased. These deviations from the normal are similar to those observed in pulmonary congestion or edema and denote a loss of elasticity and expansibility favoring the occurrence of exertional dyspnea. The arterial blood saturation is usually normal at rest but, during exertion, a significant lowering becomes apparent.

The importance of hemoglobin in the transport of carbon dioxide is reviewed, the decreased availability of hemoglobin as a buffer in carbon dioxide transport in anemia is compensated by the increased ventilation of the blood in the lungs, rendering the arterial blood somewhat alkalotic. The red cells also play an important role in regard to the respiratory enzyme, carbonic anhydrase. In the anemias due to blood loss, malnutrition, chronic infection, uremia, or leukemia, the blood carbonic anhydrase activity is parallel to the decrease in hemoglobin level leading to a deficiency not only of oxygen carrying capacity but also a decreased ability to absorb carbon dioxide from the tissues and to release it in the lungs. The following factors, many of which are closely interrelated, are operative in the production of dyspnea in anemic patients: the increased respiratory minute volume, the decreased vital capacity and its subdivisions, the abnormalities in carbon dioxide transport and dissociation, the reduced arterial oxygen capacity and the decreased blood oxygen saturation during effort, and the frequently observed elevated blood lactic acid values.

The symptoms and signs exhibited by anemic patients, including palpitation and breathlessness on exertion, tachycardia, cardiac dilatation and hypertrophy, are described. In addition to an apical systolic murmur, other systolic and diastolic murmurs are occasionally heard. The arterial blood pressure is frequently lowered in anemia, the venous pressure is generally within the limits of normal. Electrocardiographic abnormalities occur in approximately one-quarter of anemic patients but are minor and not specific in character.

The occurrence of angina pectoris, congestive failure, and intermittent claudication in some patients with the development of anemia, and disappearance of these conditions as the anemia is alleviated, is discussed with particular reference to the underlying physiologic mechanisms.

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# IRREVERSIBLE TOXIC "INCLUSION BODY" ANEMIA

## A RARELY RECOGNIZED SYNDROME, CLINICAL AND EXPERIMENTAL STUDIES

By M. H. FERTMAN, M.D., AND CHARLES A. DOAN, M.D.

A PATHOLOGIC entity, apparently rarely recognized, and unreported in recent American and British hematologic clinical literature, is a form of refractory anemia characterized by peculiar "inclusion bodies" in the circulating red blood cells. First reported by Heinz in 1890<sup>1</sup> and noted by Ehrlich in 1892<sup>2a</sup> in experimental poisonings with pyrodine, dinitrobenzol and other related compounds, "Heinz Inner-Kopern" may be readily identified in plain dried blood films, and are described in wet preparations, stained with brilliant cresyl blue or Nile blue sulphate, as deep blue, eccentrically placed spheres of varying size and number within the erythrocytes.<sup>3</sup> They are readily distinguished from Howell-Jolly bodies, siderocytosis, and classic reticulocytosis by their characteristic appearance, distribution and staining reactions.<sup>4</sup> They have gone undetected in most routine clinical laboratories probably because in the usual Wright-Giemsa stained and mounted blood films prepared for microscopic study they are difficult to demonstrate.

Our own attention has been currently focused upon this phenomenon by the discovery of its occurrence in the blood of an elderly physician with an unexplained refractory anemia, which terminated fatally despite the use of all available therapeutic measures.<sup>20</sup>

### CLINICAL OBSERVATIONS

Dr. M., #4410193, a 71 year old white male physician, was admitted to the Hematology Service, University Hospital with complaints of weakness, anorexia and nausea of four weeks duration. Attacks of angina pectoris of increasing severity and frequency had been noted for approximately four years. Eight weeks prior to this admission the patient first began to experience some dyspnea, orthopnea and moderate pedal edema.

Examination revealed an obese elderly male showing marked pallor of the skin and mucous membranes with a slight icteric pigmentation. His tongue showed no atrophic changes, and lymphadenopathy was nowhere apparent. The chest was increased in the antero-posterior diameter and fine scattered rales were heard in both lung bases. The heart was moderately enlarged to the left on percussion. A soft blowing apical systolic murmur was present. The blood pressure was within normal limits. The liver edge was palpable 6 cm. below the right costal angle, the spleen was not enlarged. Rectal examination revealed a diffusely enlarged firm prostate with no tenderness and no nodules. There was slight pitting edema of the ankles. Vibratory and position sense and deep tendon reflexes were physiologic.

The patient was temperature-free throughout his entire course in the hospital except for one transitory, mild thermal post-transfusion reaction.

Electrocardiograms demonstrated sinus tachycardia and low voltage with evidence of myocardial damage. X-ray examination of the chest showed moderate enlargement of the heart with diffuse densities in both lung fields, suggestive of some cardiac congestion. Roentgenographic examination of the stomach and lower gastrointestinal tract showed no evidence of organic pathology. X-ray plates of the skull, long bones, and bony pelvis were entirely normal.

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The admission blood study revealed 2,331,000 red cells per cu mm, 5.6 grams of hemoglobin per 100 cc, and 16,100 white cells with 83 per cent mature motile neutrophils and 13 per cent normal, small lymphocytes. Serial urinalyses and kidney function tests were consistently normal. The sedimentation rate, blood phosphorus and phosphatase, and blood urea nitrogen were within normal limits. The test for Bence-Jones protein in urine and plasma were repeatedly negative. The hippuric acid excretion was only 1.88 Gm, prothrombin time 58.8 per cent, and blood proteins 4.68 mg with 2.83 mg albumin and 1.96 mg globulin. Gastric analysis revealed a normal amount of free hydrochloric acid, and stool examinations showed no occult blood.

During his fifty-nine days of observation in the hospital, the patient was given transfusions of whole blood, and washed, resuspended red blood cells, totaling 6 liters. His red cell count ranged between 1,810,000 and 3,980,000 per cu mm. Because of the macrocytic anemia with a megaloblastic bone marrow, he was given a therapeutic trial of a concentrated form of liver extract, reticulogen, 20 units daily for seven days. This was later repeated for a ten day period. Ferrous gluconate, 5 grains three times daily was administered for fifteen days in an effort to correct the hypochromia. The patient was digitalized and received aminophyllin for his cardiac status. Other supportive treatment, included high vitamin B complex supplement.

The reticulocyte count, which was 14.8 per cent at the time liver extract was first instituted, rose to a peak of 22.8 per cent after seven days, but was not followed by any significant increase in the total circulating red blood cells. Irrespective of therapy, the reticulocytes varied between 3 per cent and 15 per cent throughout the clinical course of the anemia.

On the twenty-third hospital day the mature red blood cells, stained with brilliant cresyl blue, were first observed to contain atypical inclusions, distinguishable from the regular reticulum. As many as 13 per cent of the erythrocytes contained these bodies. Inclusion bodies were noted from then on consistently in all daily preparations, in numbers varying from 1 to 16 per cent. How long these inclusions may have been present in the patient's circulating red cells prior to their detection is a matter of conjecture.

The patient was resurveyed with reference to a possible toxic etiology for his refractory inclusion body anemia. Attention was focused on the 500  $\frac{1}{4}$  grain erythrol tetranitrate tablets taken orally over the preceding year for his angina pectoris. Except for the rare use of nitroglycerine in cardiac crises no other drug had been taken. There was no history of food idiosyncrasies or other allergic sensitivity. The patient, a physician, stated emphatically that he had never been seriously ill or anemic in a long, healthy life until the present illness.

The patient was discharged at his own request on the fifty-ninth hospital day. At this time his peripheral blood showed 3,160,000 red cells per cu mm, with 0.6 per cent reticulocytes, 7.1 grams of hemoglobin and 16.2 per cent "inclusion body" erythrocytes. His white count had fallen to 3100 per cu mm, with 44 per cent neutrophils, 8 per cent eosinophils and 48 per cent normal lymphocytes. His platelet count was always adequate.

The anginal attacks continued with frequency and severity despite bed rest, digitalis and aminophyllin. Pallor, weakness and physical debility were progressive. The red blood cells continued to show marked macrocytic hypochromia, polychromatophilia, anisocytosis, poikilocytosis, and the presence of inclusion bodies. Fatal termination occurred one month after discharge from the hospital. Post mortem examination was refused.

### CYTOLOGIC STUDIES OF THE PERIPHERAL BLOOD AND BONE MARROW

The peripheral blood showed extreme, bizarre, poikilocytosis and anisocytosis with large individual macrocytes and hypochromia. The reticulocytes were not unusual in appearance, as many as 14.8 per cent being found in the peripheral blood prior to liver therapy. No antianemic hematopoietic liver, iron, vitamins B and C, had any significant effect either on erythropoiesis or the hypochromia. Platelets occurred singly and in large clumps, and, occasional individual thrombocytes showed some qualitative changes, which included anisocytosis with sparse granulations (giant platelets). No pathognomonic alterations in the white blood cells were observed.

The bone marrow on repeated examinations appeared, grossly and microscopically, to be markedly hyperplastic. The erythroid elements were chiefly responsible for the cellular hyperplasia with a "left shift" to early erythroblasts and megakaryoblasts. There was also the hypochromia of iron deficiency. The myeloid elements showed a moderate "left shift," with more than the usual number of qualitatively normal myelocytes B but no myeloblasts. There was an increase in small, normal young megakaryocytes. No foreign tumor cell invasion was seen. There were scattered phagocytic clasmatocytes. "Inclusion bodies" were observed rarely in the immature erythroid elements in our specimens of bone marrow.

On examination of the patient's blood with brilliant cresyl blue, there were found, in addition to the classic reticulocytes, certain inclusion-containing mature red cells. With this stain, the inclusions appeared as blue green globules of irregular shape, varying in size from barely perceptible dots to spheres almost 2 micra in diameter. They occurred sometimes singly, sometimes in great numbers, within the cell. They were most often concentrated at or near the cell membrane, and on occasion were seen in various stages of extrusion from the cell. They were found to be lysis-stabile and resistant.

The inclusions could be seen in unstained living blood films as refractile yellow tinted bodies. In dark field examination they appeared highly refractile, globular and irregular, similar in general appearance and distribution to the inclusions seen in bright field. They were observed best in wet-mount preparations with brilliant cresyl blue stain, they were less clearly seen in fixed brilliant cresyl blue stained smears. Janus green with neutral red stain in supravital preparations readily revealed these "bodies." The inclusions were not apparent in fixed preparations stained with Wright or Wright-Giemsa dyes or with the Prussian blue iron technique.

The staining properties and morphology of these particular "inclusion bodies" distinguish them from Howell-Jolly bodies and siderocyte inclusions.<sup>5</sup> Howell-Jolly bodies are readily stained in Wright and Giemsa fixed preparations and appear to be dark reddish blue dots. The siderocytes are characteristically identified with the Prussian blue reaction, which leaves a heavy deep blue iron precipitate within the red blood cell.

#### EXPERIMENTAL OBSERVATIONS

Following the discovery of "inclusion bodies" in the patient's erythrocytes, it was determined that erythrol tetranitrate was the only drug which had been taken for many months prior to and coincident with the development of the anemia, and to which the patient may have developed an idiosyncrasy.

Since this peculiar anemia persisted and continued to progress even several weeks after the presumed toxic agent was discontinued, some permanent irreversible damage must have been suffered by the erythropoietic tissues. Experimental procedures were therefore undertaken in an attempt to further identify the etiologic agent and establish the mechanism involved in this fatal anemia.

#### EXPERIMENT I EFFECT OF THE PATIENT'S PLASMA UPON NORMAL RED CELLS IN VITRO

Preliminary in vitro studies were conducted to determine whether there were any toxic substances present in the fresh whole plasma of the patient which would



affect normal human red blood cells. Venous blood was obtained from a normal subject whose blood-grouping and Rh type were the same as the patient's. These normal cells were separated and resuspended in the plasma obtained from the patient and kept in a refrigerator at 10 C. Daily examination with brilliant cresyl blue over a five day period failed to reveal the development of any "inclusion bodies" in the borrowed red blood cells.

Animal experimentation was then invoked, utilizing two fundamental approaches (1) transfer of laked blood and fresh plasma was made by infusion from the patient into rabbits, (2) both rabbits and cats were subjected to erythrol tetranitrate and allied compounds and the red blood cells studied for "inclusion bodies."

#### EXPERIMENT 2. EFFECT OF THE PATIENT'S PLASMA AND LAKED RED CELLS UPON THE RABBIT

Since the patient's red blood cells contained the abnormal "inclusion bodies," it was felt that a toxic factor might be found either in the patient's plasma or in his red cells.

"Inclusion bodies" did not appear in the erythrocytes of the rabbits which received intravenous injections either of plasma or of laked red blood cells from the patient. R 1A and R 2A each received one 10 cc injection of plasma and the blood was followed for four days. R 1 and R 2 received two injections of plasma (5 and 30 cc and 10 and 20 cc, respectively) on consecutive days and died within thirty to forty-five minutes after the second dose. R 3 and R 4 received three intravenous injections of laked blood (5, 10 and 5 cc, and 1 cc, 15 cc, and 5 cc respectively). R 4 died immediately following its third injection. No significant postmortem changes were noted in the spleen, bone marrow or other organs of the three rabbits which died.

#### EXPERIMENT 3. EFFECT OF SODIUM NITRATE AND ERYTHROL TETRANITRATE UPON ERYTHROPOIESIS IN THE RABBIT

Two rabbits (R 1A, R 4A) were then given massive doses of sodium nitrate every day subcutaneously. Necrosis at the site of injection necessitated substitution of the oral route via the stomach tube on the fifth day. In R 4A the dose was doubled from 100 to 200 mg /Kg on the fifth day and death occurred on the seventh. In R 1A the dose was increased from 500 to 1000 mg /Kg on the fifth day and to 2000 mg /Kg on the tenth. Death occurred on the eleventh day.

R 3 and R 2A received 50 to 300 mg /Kg doses of erythrol tetranitrate via stomach tube daily over a period of more than two weeks. R 3 was followed for three days after completion of a sixteen day course of the drug, while R 2 died on the fifteenth day.

Daily examination of the blood of these rabbits receiving either sodium nitrate or erythrol tetranitrate even in massive doses revealed no "inclusion bodies." No significant postmortem changes were observed in the hematopoietic organs in the three rabbits which died.

## EXPERIMENT 4 EFFECT OF ERYTHROL TETRANITRATE UPON THE MONKEY

A *Macacus rhesus* monkey received three 180 mg /Kg doses of erythrol tetranitrate by stomach tube on the second, fourth and tenth days of observation. During daily observations for twenty-three days, a mild anemia was precipitated, without the development of "inclusion bodies," from which there was prompt recovery with cessation of the drug.

## EXPERIMENT 5 EFFECT OF SODIUM NITRATE AND ERYTHROL TETRANITRATE UPON THE CAT

More suggestive results were obtained in experiments with cats. "Inclusion bodies" appeared within twenty-four to seventy-two hours in the red blood cells of all cats receiving either sodium nitrate or erythrol tetranitrate.

C 1 received a single subcutaneous injection of 1000 mg /Kg of sodium nitrate and C 2 injections of 500 mg /Kg on two consecutive days. In both, 'inclusion bodies' up to 9 and 10 per cent appeared within forty-eight hours after the initial dose and then gradually declined as the drug was eliminated.

Three cats (C 9, C 3, C 1) received oral doses of powdered erythrol tetranitrate, which was thoroughly mixed with raw meat. C 9 was given a single dose of 250 mg /Kg and showed at seventy-two hours 21 per cent of the circulating erythrocytes with typical 'inclusion bodies,' and at ninety-six hours 28 per cent, with a fall of 2,000,000 in red cells during this period. During the ensuing fifteen days the "inclusion body" erythrocytes gradually declined to zero per cent with a recovery in the red cells. In C 3, 200 mg /Kg of erythrol tetranitrate was administered on the fifth, sixth, eleventh and twenty-seventh days of observation, and 300 mg /Kg on the fifteenth and seventeenth days. A rare red cell containing 'inclusion bodies,' 1 per cent, was observed within twenty-four hours of the original dose. A peak of 15 per cent was attained after the last dose, associated with a drop of 1,000,000 in the total circulating red blood cells.

In C 1, weighing 2.8 Kg, erythrol tetranitrate was administered beginning nine days after one dose of 1000 mg /Kg of sodium nitrate. The 'inclusion bodies' had risen to 10 per cent within forty-eight hours of the sodium nitrate dosage and had gradually fallen again to 2 per cent by the time the first dose of erythrol tetranitrate (150 mg /Kg) was administered. The next day, another 150 mg /Kg of erythrol tetranitrate was given, followed by doses of 75 mg /Kg on the 3, 4, 5, 6, 11 and 12 days of observation. The 'inclusion bodies' rose to 26 per cent within the first seventy-two hours of the erythrol tetranitrate administration and varied from 11 to 28 per cent through the thirteenth day. The hemoglobin decreased from 13.3 to 11.2 Gm during the period. With discontinuance of the drug on the twelfth day, the 'inclusion bodies' declined to 5 per cent by the sixteenth day.

In the cats receiving sodium nitrate or erythrol tetranitrate, the concentration of 'inclusion bodies' did not vary directly with the dosage of the drug under the conditions of the experiment. With the cessation of the drug, the number of 'inclusion bodies' was noted to decrease promptly and to disappear entirely eventually. A decrease of between one and two million in the red cell count was



1



2



3



4

observed to occur in all cats from the time the first nitrate was administered. In C 9 recovery in the red cell count was soon evident with cessation of the drug.

#### EXPERIMENT 6 EFFECT OF MANNITOL HEXANITRATE ON CAT BLOOD

Less spectacular results were observed in two cats (C 6, C 10) fed mannitol hexanitrate, pulverized in meat. Despite relatively large doses of the drug (165 to 330 mg/Kg) only small percentages of "inclusion bodies" (1 to 6 per cent) were observed in C 10, and the red cell count showed no significant change.

An *old* cat (C 6), with an occasional "inclusion body" erythrocyte (0.5 per cent) even prior to nitrate administration, developed a maximum of 12 per cent of these after nine successive doses of mannitol hexanitrate (80 to 160 mg/Kg), the loss in circulating red cells totaling 4,000,000, hemoglobin 3 Gm over a thirty-four day period of observation. The rare occurrence of "inclusion bodies" in the red cells of older animals has been reported elsewhere in the literature.<sup>5</sup>

#### EXPERIMENT 7 EFFECT OF SULFANILAMIDE UPON CAT RED CELLS

Sulfanilamide was only questionably effective in the production of "inclusion bodies" under the conditions of this experiment.

In C 2, which had shown up to 9 per cent "inclusion bodies" with two 500 mg/Kg doses of sodium nitrate, the highest concentration of "inclusion bodies" observed after ten daily 400 mg/Kg (oral) doses of sulfanilamide was 4 per cent. Results were negative in an *old* cat, C 7, which showed no significant increase in "inclusion bodies" over a 5 per cent concentration demonstrable prior to sulfa drug administration, nine successive doses of sulfanilamide (800 to 1600 mg/Kg per dose). The red cell count and hemoglobin showed no consistent trend during the period of observation in either cat.

#### STAINING PROPERTIES OF THE "INCLUSION BODIES" IN CATS

The staining properties of the "inclusion bodies" observed in the erythrocytes in cats appeared to be similar to those in our patient. The "inclusion bodies" were seen readily in unstained preparations and in supravital preparations stained with brilliant cresyl blue, methylene blue and Janus green with neutral red (fig. 7). They were also seen, but less readily, in fixed preparations with brilliant cresyl blue. They were not apparent in preparations stained with Wright, Wright-Giemsa or with the Prussian blue reaction.

Certain differences were noted in the cellular reaction in the cats as contrasted with the patient. In the animal studies only a minor degree of poikilocytosis and aniso-

Figs. 1, 2. Brilliant cresyl blue supravital staining of the erythrocytes in patient's blood. Inclusion bodies as seen in bright field under oil immersion.

Figs. 3, 4, 5. Similar preparations of the peripheral blood from the patient as seen under dark field oil immersion conditions. Note highly refractile inclusion bodies. Figure 4 shows a separated inclusion body indicating the integrity of these Heinz inner körpers apart from the erythrocytes. Figure 5 identifies the peripheral location of these bodies at the cell surface.

Fig. 6. A bright field illustration of inclusion bodies from the patient's blood. Compare and contrast with similar bodies appearing in the blood of Cat 2 following erythroltetranitrate oral medication, FIGURE 7

cytosis was noted, whereas in the human blood, these changes were extreme. Perhaps this reflects the more profound hematologic disintegration in the patient, due to the much longer period of exposure to the drug. It was also observed that the "inclusion bodies" in cat blood tended to remain globular while "inclusion bodies" in the patient's blood were more bizarre in shape (figs 6-7). In addition the "inclusion bodies" observed in the cats under conditions of the experiment occurred in fewer numbers within each red cell than those observed in the patient.

The "inclusion bodies," both in the patient and in cats, were noted in various phases of extrusion from the red blood cell (figs 4,5,7). They were found within the cell, close to the periphery, producing a bulge in the red cell membrane and also lying free outside the red cell. "Inclusion bodies" in both human and cat blood were found to be lysis-resistant.

A specimen of cat blood, demonstrated to contain "inclusion bodies," was taken and selectively centrifuged to obtain a highly concentrated specimen of these "bodies." Spectroscopic examination of this specimen revealed no hematorporphyrin. This evidence, while suggestive, is not conclusive.

Bone marrow examinations were made in cat #1 after erythrol tetranitrate in massive amounts. Unlike the bone marrow of the patient, who had received the drug in small doses over a long period of time, the erythroid elements showed no maturation arrest and appeared normal except for the occurrence of small punctiform perinuclear inclusions in a few normoblasts.

## DISCUSSION

Peculiar "inclusion bodies" were observed in the red blood cells of a 71 year old male physician who exhibited a severe anemia, which did not respond to either intensive iron or liver therapy nor to a high protein, high vitamin diet. Although folic acid as such<sup>6</sup> was not available for treatment, large amounts of vitamin B<sub>12</sub> complex were given. There was no clinical evidence of any specific nutritional deficiency and there was no response to liver. The "inclusion bodies" were noted in the unstained preparation and were stained readily with supravital wet-mount technic, using brilliant cresyl blue and other reticulocyte stains. They appeared as blue green irregular globules, occurring singly and in numbers, within the mature red cells, and taking a position at the periphery. They were also seen in various stages of extrusion from the red cells and were noted to be hemolysis-resistant. The "inclusion bodies" were distinguished from Howell-Jolly bodies and from siderocyte inclusions. These various staining and morphologic characteristics were found to conform with those of the "Heinz Inner-Korpern" described in the German literature<sup>1-4,7</sup> as occurring in "toxic" anemias in man and in animals.<sup>1-4,7-17</sup>

The suspected etiologic agent, erythrol tetranitrate, which the patient had taken for angina pectoris over a period of one year, was administered to cats in massive doses. Lysis-resistant "inclusion bodies," with staining characteristics similar to those found in the patient's blood, were induced. A tendency toward anemia, reversible under the conditions of the experiment, with cessation of the drug, was observed. "Inclusion bodies" were noted to a lesser extent with sodium nitrate and mannitol hexanitrate. Only minimal concentrations of "inclusion-bodies" were

produced with large doses of sulfanilamide. We can confirm the report of German investigators<sup>8</sup> that old animals show these "inclusion bodies" in a small proportion of circulating erythrocytes in the absence of any known external toxic agent.

Although erythrol tetranitrate and sodium nitrate were given in large repeated doses to rabbits, "inclusion bodies" were at no time observed. In a monkey receiving erythrol tetranitrate, a mild reversible anemia developed without demonstrable "inclusion bodies."

Cessation of drug administration to cats brought about a gradual disappearance of the "inclusion bodies" from the blood stream. Whether a longer course of drug administration would have produced an irreversible "inclusion body" anemia in the cat, such as that observed in our patient, remains unanswered.

The observance of a progressive, refractory, "inclusion body" anemia in an elderly patient, persisting even after four months' omission of the suspected toxic drug, would suggest the precipitation of an irreversible toxic alteration in the erythrocyte maturation process.

Various theories have been proposed to explain the formation, nature and significance of these "inclusion bodies." Freifeld<sup>8</sup> suggests a genetic relationship between the so-called "Randkorperchen" (corpora marginalia) and these "Inner-Korpern" of Heinz and Ehrlich, the result of an enterogenous autointoxicant. These "inclusion bodies" were originally regarded as "dead toxic protoplasm,"<sup>1,2</sup> reflecting methemoglobin or sulph-methemoglobin poisoning.<sup>9</sup> The more recent interpretation of these "Inner-Korpern," as denatured, cell-membrane proteins<sup>1,10</sup> has received support from Jung's observations<sup>7</sup> with the electron microscope, which place these "inclusion bodies" definitely in the outer layer of the cell. Frank H J Figge, Associate Professor of Anatomy, University of Maryland, has studied the experimental production of Heinz-body erythrocytes.<sup>18</sup> "When certain sulfonamides were dissolved in water administered to mice, large numbers of refractile bodies appeared in the erythrocytes. These bodies were insoluble in distilled water or 3 to 5 per cent acetic acid and made leucocyte counts difficult. They were similar to erythrocyte inclusions described originally by Heinz. Other investigators concur in this identification. It was found that a 0.3 per cent sulfanilamide solution given as drinking water induced Heinz bodies in at least 90 per cent of all erythrocytes within four to six days, while sodium sulfathiazole did not. The tendency of various sulfonamides to produce Heinz bodies appeared to parallel the tendency to induce hemolytic anemia. Further studies on the physical and chemical properties revealed that these bodies are globules of either denatured hemoglobin or cathemoglobin. Erythrocytes extrude these bodies as they form so that large numbers of Heinz bodies accumulate in the plasma as the erythrocytes become hypochromic. Heinz bodies are most easily observed in unstained, unmounted blood smears and disappear when examined in oil, balsam, or other mounting media. These globules of hem-containing protein, denatured within the cell by drugs, have been studied in detail because such a reaction is of interest both from the standpoint of cancer research and the mode of action of sulfonamide drugs. Dr Figge in a recent personal communication<sup>19</sup> further states: 'I still do not know the exact mechanism which is responsible for the production of these bodies. They can be produced in

small numbers by such diverse agents as cobalt, paraminobenzoic acid and acetanilide. I suspect that they are probably formed as a result of therapy with numerous other compounds but go undetected because blood is usually prepared for examination with some mounting medium. As you have probably noticed, these bodies are much more easily observed in plain dried blood films examined under the high dry objective."

The conception of 'Heinz Inner-Korpern' as nuclear fragments has not been confirmed, though Figge states "this protein, which is denatured, resembles in some respects a nucleoprotein."

Schilling, who noted 'all transitions' between typical Howell-Jolly bodies and the 'Inner-Korpern' following splenectomy,<sup>9</sup> rejected this simple explanation.<sup>11</sup> The appearance of "inclusion bodies" in the peripheral blood of various normal animals following splenectomy<sup>3, 8, 9, 11</sup> and of humans with splenic hypoplasia, subsequently demonstrated,<sup>1</sup> bears out the hypothesis of Heinz<sup>1</sup> and of Schilling<sup>9</sup> that the normal spleen filters out the senile and damaged erythrocytes, which may contain these 'inclusion bodies.' This is further corroborated by the observation of 'inclusion bodies' (Giemsa stained Tupper preparation) as partly extra-cellular and partly extruding from the red cells in the human spleen.<sup>3</sup> It is suggested that in the absence of a normally functioning spleen the inclusion-containing red cells may appear in the peripheral blood. The administration of a specific toxic agent further increases their numbers.<sup>3</sup>

Should it be considered, then, that minimal "inclusion body" formation may occur, in association with subclinical endogenous toxins, in otherwise normal individuals, but in numbers so small that they are ordinarily withdrawn by a physiologically functioning spleen? This concept might explain the more frequent observation of small numbers of inclusion-containing erythrocytes in the blood stream of senile animals,<sup>8</sup> animals in whom the catabolic processes predominate and splenic efficiency perhaps may have diminished. In addition, there may be other states, such as dietary deficiencies, inherent specific susceptibilities and certain disease processes, which perhaps may predispose to 'inclusion body' anemia. One may also inquire as to whether the development of "inclusion bodies" may precede red blood cell destruction at some phase in anemic states other than those of "toxic" etiology. It has been shown that in poisoning with nitrates and their derivatives the extent of 'inclusion body' development parallels closely the destruction of the red blood cells.<sup>4</sup>

The known 'toxic' agents which may result in an "inclusion body" anemia in man and in animals are, in general, methemoglobin producing drugs, such as nitrobenzol,<sup>2, 9</sup> aniline,<sup>8, 9</sup> nitroglycerine,<sup>4</sup> dinitroglycol,<sup>4</sup> ethyl nitrate,<sup>4, 12</sup> sodium nitrate,<sup>12</sup> nitrobenzol and toluol derivatives.<sup>8, 13</sup> Even in the absence of a known "toxic" agent, as in an hemolytic anemia observed in rats following splenectomy, "inclusion bodies" have been reported in association with methemoglobin formation.<sup>8</sup>

Nevertheless, a "cause and effect" relationship between methemoglobin and "inclusion body" erythrocytes has been questioned. The appearance of "inclusion bodies" in the peripheral blood does not always follow methemoglobin formation.<sup>8, 11</sup>

In one investigation, the "agent," rather than the methemoglobin per se, was found to be the more important factor in the concentration of "inclusion bodies" in the blood. Thus, the nitrates, which form less methemoglobin than the nitrites, have produced many more inclusion bodies.<sup>12</sup>

Observations<sup>8</sup> in eleven patients with acute poisoning from aniline or nitroderivatives have revealed that "inclusion bodies" appear only several hours after methemoglobin is formed. Moreover, several more hours may elapse, as noted in a schizophrenic who had swallowed one-half glass of aniline, before these bodies become prominent and involve a maximum number of the mature circulating red cells, even to 100 per cent. In this patient, massive hemolysis of the red blood cells was noted on the fifth day and "inclusion bodies" were seen extruding in various stages from hemolyzing red blood cells. Immediately after, there occurred a reticulocyte crisis with normoblastosis.<sup>8</sup>

Since 1943 there have appeared several reports in the German literature of sulfonamide drug anemia preceded by the appearance of inclusion-containing erythrocytes.<sup>14-17</sup> It has been advised that if more than 20 per cent appear, a severe hemolytic anemia may be forecast, and the drug should be forthwith discontinued.<sup>14</sup>

#### SUMMARY

1 "Inclusion bodies," distinguishable from the Howell-Jolly bodies, were observed in the red blood cells of a patient with a severe refractory fatal anemia, who had been receiving erythrol tetranitrate over a period of one year.

2 "Bodies" with similar staining characteristics were reproduced in cats with large oral doses of erythrol tetranitrate and other nitrates. These were generally accompanied by a temporary fall in the red cell count, followed by recovery upon withdrawal of the drug.

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# BLOOD RESPONSE AND NITROGEN BALANCE FOLLOWING LIVER EXTRACT

By RANDOLPH WEST, M D

ONE PATIENT with addisonian pernicious anemia, and one with sprue with normal gastric acidity and without diarrhea, were studied (Unit history number 442154 and 486382 respectively)

The patients were in the metabolism ward on weighed diets Urinalyses were done on 24 hour specimens, stool samples were pooled for several days and aver-

TABLE I

*Sprue*

	Day		
	1*	7	14
N balance		-11 6	+8 9 Gm per period
RBC	1 0	1 5	2 4 millions
Retics	1 8	49 0	16 0 per cent
Hgb N	26 7	59 2	79 9 Gm total
Plasma N	42 5		42 4 Gm total
Creatine		3 16	1 1 Gm per period

*Pernicious Anemia*

	Day		
	1	9†	19
N balance		-3 8	+18 4 Gm per period
RBC	2 7	2 6	3 7 millions
Retics	1 9	2 8	2 6 per cent
RBC N	88	7 6	99 Gm total
Plasma N	36		31 Gm total
Creatine		0 781	0 067 Gm per period

\* Liver extract 150 units parenterally, day 2

† Liver extract 45 units parenterally every two days from day 9

aged Plasma volume and red cell volume were done by T-1824 and venous hematocrit, serum proteins by the Howe method The main results are given in table 1

In the sprue patient the hematologic response took place while the patient was in negative nitrogen balance, in the pernicious anemia case positive N balance and blood response coincided

The plasma N did not increase significantly, the rise in hemoglobin N represents a transfer from bone marrow to peripheral circulation

It is of interest that in both cases positive balance was established and creatinuria

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lessened In the pernicious anemia patient this occurred without change in N or caloric intake, in the sprue case appetite made increased diet essential to satisfy the patient

Mosenthal<sup>1</sup> in 1918 showed that the forced feeding of a diet rich in meats restored nitrogen balance in pernicious anemia

The present studies were undertaken to determine whether the positive nitrogen balance appeared before the hematologic response following liver therapy If this had occurred it might indicate that an important site of action of liver extract was on the gut wall This, however, was not the case, and liver extract presumably acts directly on the immature red cells in the marrow cavity

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# THE PATHOGENESIS OF ANEMIA IN ACUTE GLOMERULONEPHRITIS ESTIMATIONS OF BLOOD PRODUCTION AND BLOOD DESTRUCTION IN A CASE RECEIVING MASSIVE TRANSFUSIONS\*

By CHARLES P. EMERSON, M D

ANEMIA is one of the familiar manifestations of Bright's disease and a frequent complication of uremia, irrespective of the etiologic factors responsible for renal failure <sup>1-3</sup> A peculiarly intimate association exists between anemia and glomerulonephritis, in relation to which this hematologic sign is of diagnostic and prognostic importance <sup>4-5</sup> Conclusions regarding its pathogenesis are based on the experience of numerous investigators, who have emphasized the consistent lack of signs indicating excessive blood loss or blood destruction, and have succeeded in correlating the occurrence and severity of this anemia with the degree and duration of associated azotemia <sup>2-4, 6-7</sup> Hence, the anemia associated with nitrogen retention is generally regarded as an example of erythropoietic failure Furthermore, its refractoriness to erythropoietic stimulation with iron or liver therapy has been interpreted <sup>8-11</sup> as evidence that blood production in patients with renal decompensation is retarded in consequence of 'toxic inhibition of the bone marrow' by retained nitrogenous metabolic products

This hypothesis, although possibly correct, as a premise mainly deduced through analogy, by inference and by exclusion bears particular scrutiny, little or no evidence of a positive and unequivocal sort having been marshalled in its support The toxic metabolite presumed to be implicated has thus far escaped identification, and of all of the numerous chemical agents recognized as bone marrow depressants there is none known to exert comparable effects on the bone marrow or peripheral blood Finally, it may be objected that the hematologic data cited from case reports in support of this concept, including descriptions of reticulocytosis and alterations of bone marrow histology, <sup>5, 12-14</sup> in some instances suggest an enhancement, rather than a depression, of erythropoietic activity in patients with nephritis and anemia

Transfusion studies, designed to permit an estimation of the survival of injected donor red cells, have contributed valuable information relative to the pathogenesis of various types of anemia, particularly those associated with certain hemolytic syndromes <sup>15-16</sup> This technic of investigation, employing serial measurements of the circulating blood volume in addition to selective agglutination counts, was applied in the study of a patient with an initial attack of early acute glomerulonephritis, who, presenting signs of moderate azotemia and progressive anemia, was adjudged particularly suitable as a subject for detailed hematologic investigation Data were accordingly obtained which served as a basis for the relative evaluation of blood production, blood loss and blood destruction as factors possibly implicated in the development of his anemia Appreciating the limited significance of the results obtained, which, pending confirmation from comparable investigations can

\*The data utilized in this case report were obtained while on active duty with the Fifth (U S) General Hospital in the European Theater of Operations

hardly be evaluated in relation to other patients with Bright's disease, the observations are nevertheless considered of sufficient interest to warrant description in the form of an individual case report

### CASE HISTORY AND INITIAL OBSERVATIONS\*

A 27-year-old, white, American enlisted soldier was admitted to the hospital complaining of progressive swelling of the legs. Two months before entry he had contracted an acute pharyngitis which completely subsided in the course of several days. Thereafter he experienced persistent weakness and unusual fatigability. Two weeks before admission he became aware of painless swelling of his lower extremities, which increased, and, together with symptoms of general malaise, headaches and anorexia, occasioned his entry to the hospital.

The physical findings on admission were those of a well-developed male with pallid complexion and obvious pitting edema of the lower extremities. His body temperature was normal, arterial pressure 190/130 mm Hg, height 168 cm, weight 73.4 Kg (13 Kg in excess of his average weight prior to the present illness).

*Initial laboratory data.* Urinalysis: Specific gravity 1.015, albumin 4+, sediment (uncentrifuged) containing 15-20 r b c, 2-4 w b c and numerous casts, granular and cellular per high power field. Blood hemoglobin concentration, 13.1 Gm per cent, red cell count, 3.98 million per cu mm, hematocrit reading, 36.7, leukocytes, 8,200 per cu mm, with normal differential count, platelets, 294,000 per cu mm. Erythrocyte osmotic fragility normal, sedimentation rate (Westergren) 17 mm in one hour. Blood urea nitrogen concentration, 25 mg per cent, total serum protein concentration, 4.4 Gm per cent. Bleeding time (Duke) 3½ minutes, clotting time (Lee-White) 8½ minutes. Stool examinations were negative for occult blood. The initial throat culture contained beta hemolytic streptococci, this organism failing to be demonstrated on re-examination after eight days.

### METHODS OF STUDY

The patient was observed for a period of fifty days during which he was essentially at complete bed rest, maintained on a dietary regime restricted solely with respect to its sodium content. Penicillin, 120,000 units daily, was administered intramuscularly from the fourth to the thirteenth day. Otherwise, apart from transfusions and albumin injections subsequently to be specified, no therapeutic agents, hemopoietic, diuretic or antibacterial, were employed.

Daily observations included measurements of the arterial pressure, body weight, fluid intake and urine volume. Urinalyses were performed daily, which included, after the eighth day, a quantitative (Esbach) estimation of the total urine albumin excretion. Blood hemoglobin concentrations and icterus indices were determined with Klett photoelectric technics. Blood urea nitrogen was measured colorimetrically after urease digestion and nesslerization, and total protein concentrations by the procedure of Phillips and Van Slyke.<sup>17</sup> Plasma volume determinations, employing T-1824 dye, were performed by a modification<sup>18</sup> of the method of Gibson and Evans.<sup>19</sup> Calculations of the circulating red cell volume and total blood volume were based on the plasma volume and hematocrit values, these computations involving a correction factor of -15 per cent applied to the calculated red cell volume to compensate for the relatively constant disparity between the large vessel hematocrit and the total body hematocrit.<sup>20</sup>

Group-O donor blood, freshly drawn into acidified glucose-citrate anticoagulant

\*Identifying initials of the patient have been deleted here and in the table and figures at the request of the Technical Information Office of the Surgeon General's Office.

solution, was employed in the first course of transfusions, in preparation for the second series red cells from freshly obtained group-O blood were washed once and resuspended in 0.85 per cent saline solution. At intervals following transfusions the concentration of donor cells was determined, from these data it was possible to calculate the total volume of circulating donor cells and group-A recipient's cells. Selective agglutination counts were performed by modifications of the Ashby technic, utilizing dried anti-A grouping serum, a procedure that has been successfully applied in other investigations<sup>21, 22</sup> and has recently been evaluated by Young<sup>23</sup>

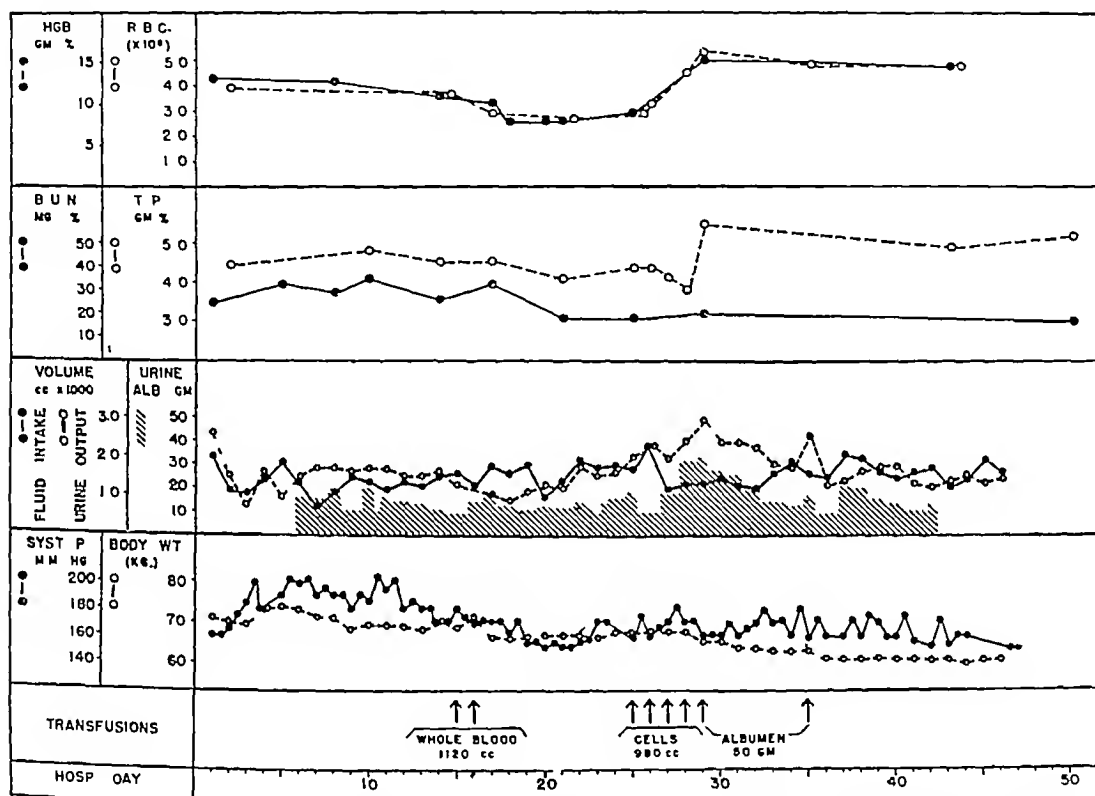


FIG 1 Hematologic, blood chemical, and clinical data on a case of acute glomerulonephritis receiving infusions of whole blood, red cells and albumin

### COURSE

The hematologic findings and metabolic data obtained in this case are charted in figure 1 which depicts the observed fluctuations in the hemoglobin concentration, red cell count, blood urea nitrogen, total serum protein concentration, fluid balance, total albumin excretion, body weight and systolic arterial pressure

It was evident on the fifteenth day that the patient's anemia, which was of a normocytic, normochromic type, was progressing in severity, the hemoglobin concentration having decreased from 13.1 to 11.2 Gm per cent and the hematocrit reading from 36.7 to 30.8 since admission to the hospital. Blood volume measurements (table 1 and figure 2) indicated a total circulating red cell volume of 1080 cc representing a calculated deficit of approximately 900 cc or 45 per cent, relative to

the expected volume for an average normal male of his stature. The total blood volume was likewise deficient (approximately 20 per cent), this decrease being en-

TABLE 1—*Blood Studies in the Course of Transfusion Therapy*

Hosp Day	Hgb	Veno- us Hct	Donor Rbc (O)	Plasma vol- ume	Total blood vol	Red cell volume			Retic	Icterus index	Blood ure-N	Plasma proteins	Blood transfusions
						Total	Group A	Group O					
	Gm	%	%	cc	cc	cc	cc	cc	%		mg %	Gm %	
1	13.1	36.7	0	—	—	—	—	—	—	—	25	4.4	Rbc (o) 470 cc., Plasma, 650 cc.
14	11.2	30.8	0	2880	3960	1080	1080	0	1.2	6	26	4.5	
15-16													
17	10.0	28.5	41	3270	4370	1100	650	450	13.8	11	32	4.5	Rbc (O), 260 cc. Rbc (O) 245 cc. Rbc (O) 230 cc. Rbc (O) 240 cc.
21	8.6	23.8	37	—	—	—	—	—	8.9	—	17	4.1	
25	9.3	27.1	38	2770	3650	880	550	330	11.5	6	17	4.3	
26	—	33.1	48	—	—	—	—	—	—	6	—	4.4	
27	—	36.6	52	—	—	—	—	—	—	8	—	4.1	
28	—	41.6	57	—	—	—	—	—	—	9	—	3.8	
29	15.3	43.7	57	2450	4060	1610	690	920	—	14	20	5.5	
43	14.8	40.2	52	2880	4540	1660	800	860	—	6	—	4.9	
50	—	41.4	48	—	—	—	—	—	—	—	16	5.3	
Values expected in normal male, ht 168 cm (20, 24)				2850	4850	2000							

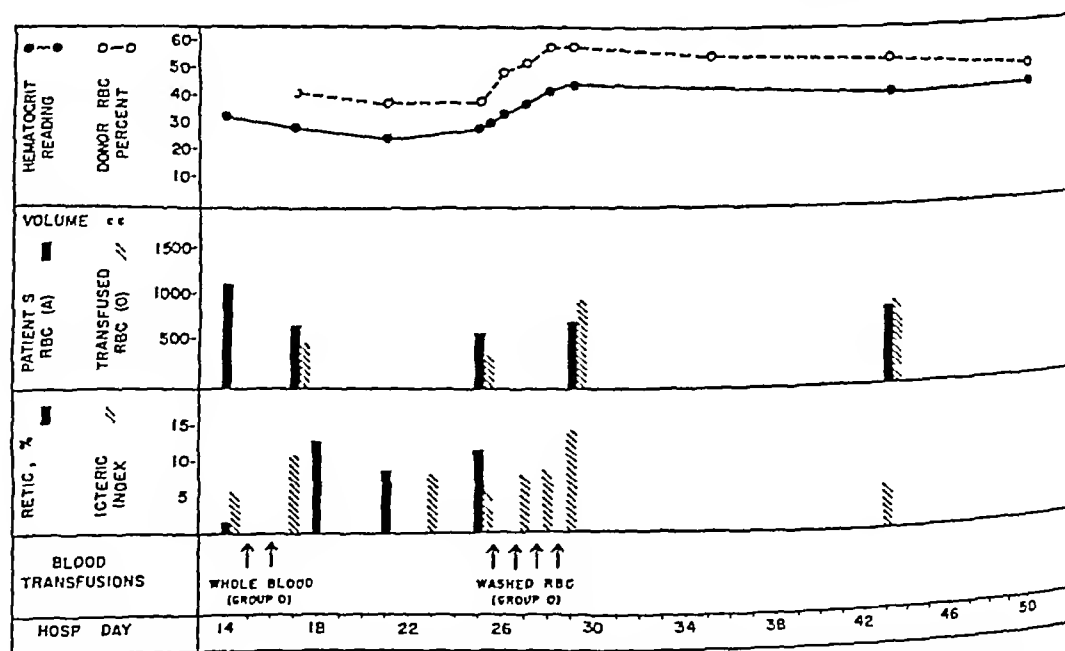


FIG. 2. The influence of blood transfusions on anemia associated with acute glomerulonephritis

tirely accounted for on the basis of the decrease in red cell volume, the plasma volume being within normal limits. Blood loss via the gastrointestinal and renal

tracts had been insignificant, hematuria continuing to be of microscopic degree, and only an occasional stool containing a trace of occult blood during the entire period of observation.

*First transfusion series* On the fifteenth, and again on the sixteenth hospital day the patient received group-O whole blood totalling 470 cc of red cells and 650 cc of plasma. A mild febrile reaction followed on each occasion and, at the conclusion of the second injection, he experienced transient severe lumbar aching pain. The spleen promptly became enlarged and remained palpable for two weeks. Hemoglobinemia and hemoglobinuria did not occur, but there developed for the first time a mild transient icterus, a relative decrease in urine output and an increase in albumin excretion.

Blood volume studies and selective agglutination counts performed on the day following the second transfusion demonstrated that, whereas the injected red cells had survived *in toto*, a destruction of the patient's group-A cells had occurred, this loss approximately equalling the volume of transfused erythrocytes (table 1 and fig. 2). Thereafter, during the nine day interval between the first and second series of transfusions, the red cell volume continued to diminish, the patient's cells decreasing by 100 cc (15 per cent) and the donor cells by 120 cc (27 per cent). This progression of anemia developed despite the onset of a persistent reticulocytosis which occurred abruptly following transfusion (fig. 2).

*Second transfusion series* Between the twenty-fifth and twenty-ninth hospital days the patient was transfused with saline-washed group-O cells derived from 2000 cc of whole blood, all plasma having been removed. The total red cell volume was thereby increased to 1610 cc, or approximately 80 per cent of the expected normal value. There was a recurrence of mild transient icterus which, on the basis of selective agglutination counts, was apparently due to the prompt destruction of those donor cells injected in the final transfusion. No destruction of patient's cells resulted and those donor cells remaining at the conclusion of this series of transfusions survived thereafter in normal fashion, less than 10 per cent being eliminated in the course of the ensuing two weeks. A progressive increase in the patient's circulating red cells was demonstrable after the twenty-fifth day, their total volume being 250 cc, or 45 per cent, greater on the forty-third day. Although no subjective symptoms were associated with the cell transfusions a definite increase in proteinuria was noted immediately thereafter, a phenomenon which had likewise followed the earlier transfusions of whole blood, and the subsequent administration of crystalline human albumin.

#### DISCUSSION

This report concerns a patient hospitalized early in an initial attack of acute glomerulonephritis with manifestations of arterial hypertension, hematuria, albuminuria and nitrogen retention, who was under continuous observation for fifty days. During the first observation period, when renal decompensation was maximal, although by no means marked, there developed a moderately severe normocytic normochromic anemia of a type commonly associated with nephritis. Two weeks following the appearance of dependent edema, the first clinical evidence of



his renal disease, the venous hematocrit reading was 36.7, a reduction of approximately 20 per cent, two weeks later the hematocrit was 30.8, approximately 30 per cent below normal, but the calculated deficit in total circulating red cell volume at this time was 45 per cent. Measurements of the plasma volume indicated that there had occurred no compensatory increase in the latter, and that the true severity of the anemia, judged solely on the basis of the red cell and hemoglobin concentrations, had been obscured as a result of a reduction in the total blood volume. Alterations of a similar character in patients with acute nephritis have been reported by Harris and Gibson.<sup>25</sup>

Rapid red cell depletion of the degree exhibited by this patient is difficult to explain solely on the basis of erythropoietic depression due to toxic inhibition of the bone marrow, to a metabolic defect, or to a nutritional deficiency. Even assuming a complete cessation of blood production the decline in the red cell volume occurred at approximately twice the expected rate<sup>26</sup> of 0.8 to 1.0 per cent per day, unless this bone marrow aplasia is considered to have occurred at the time of the antecedent pharyngitis. The latter possibility can hardly be discarded, but such an hypothesis presumes that the initial observations of hematocrit and hemoglobin concentration were misleading, the plasma volume at that time being considerably lower than when first determined two weeks later, a supposition for which there is no basis. Complete bone marrow inactivity is in any case an improbable explanation for the observed anemia, on the grounds that the reticulocytes, although not numerous (1.2 per cent) before transfusion therapy, were nevertheless present. It is of interest that the presence of reticulocytes in the peripheral blood has consistently been described in case reports published in relation to this problem, whatever interpretations may have been adduced from the hematologic data obtained.

Significant blood loss having been adequately excluded during the entire period of observation, it is inferred that excessive and uncompensated blood destruction must have been responsible for the rapid development of anemia in this case. Observations following the first series of transfusions tended to confirm this evaluation of the mechanisms involved. The patient, blood group-A, received 1000 cc of group-O whole blood in the course of twenty-four hours, a procedure which precipitated a mild hemolytic crisis, with prompt destruction of 430 cc of his own cells. Inasmuch as the donor erythrocytes quantitatively replaced the destroyed recipient cells there occurred no significant change in the severity of the anemia. An important contributing factor in this response was unquestionably the presence of incompatible isoagglutinins in the injected donor blood, the hemolytic effect of which has been previously described. Unfortunately the titer of anti-A isoagglutinins in the injected material was not determined. It may be stated, however, that no instance has been observed<sup>21</sup> in which a comparable degree of hemolysis was produced by the first transfusion of plasma or whole blood containing incompatible isoagglutinins in very high titer, hence this patient must have been unusually susceptible to the hemolytic effect of the "universal donor" blood he received. Of far greater significance are the observations pertaining to the subsequent fate of the normal donor erythrocytes which were eliminated at an average rate of 3 per cent per day, or more than three times the expected rate. Depletion of the patient's own

cell population also continued to be excessive (16 per cent per day) but their net loss occurred less rapidly, which may be explained on the basis of a sudden increase in blood production evidenced by a concomitant reticulocytosis (fig. 2)

This sudden and unexpected increase in reticulocytes, indicating enhanced erythropoietic activity immediately following transfusion, deserves particular mention. Immediately preceding this therapy the reticulocyte count was 12 per cent, immediately thereafter the percentage had increased to 13.8. The precise explanation for this phenomenon is not evident, but it is of interest that the peak reticulocytosis occurred prior to a further substantial reduction in the venous hematocrit or hemoglobin concentration, hence, the stimulus for increased bone marrow activity was not primarily an increase in bone marrow hypoxia. Moreover, inasmuch as it occurred at a time when the elevation of blood urea nitrogen was almost maximal, one is tempted to reject the hypothesis that the previous inadequacy of blood production was due to toxic inhibition of the bone marrow as a result of nitrogen retention or to other unexcreted metabolites. It is possible that the resumption of normal erythropoietic activity displayed at this time was related to the increased hemolysis provoked by the administration of incompatible isoagglutinins, that it occurred, not as a result of increased anemia, donor erythrocytes having been substituted almost quantitatively for the patient's hemolyzed red cells, but due to the stimulus of some hemopoietically effective material derived from the latter. Or the donor blood may have been the source of an erythropoietic agent, of which there had been a previous deficiency. Whatever the true explanation, blood formation proceeded thenceforth at an increased rate, although temporarily outpaced by blood destruction.

As a result of the second series of transfusions, involving the administration of plasma-free red cell suspensions, the patient's anemia was practically relieved. In the course of four days the hemoglobin concentration was increased from 9.3 to 15.3 Gm per cent, and the hematocrit reading from 27.1 to 43.7, the total red cell volume was almost doubled. A significant proportion of the injected cells were hemolyzed in the process of their preparation, or were eliminated very promptly following the injection. Nevertheless the subsequent fate of this donor blood, which survived normally, together with data indicating a progressive increase in the patient's red cell population, suggest that abnormal blood destruction had ceased, and that blood formation was occurring at a normal rate. The factors responsible for this reversion to a normal hematologic status can not be positively identified on the basis of the available evidence. It is of interest, however, in view of the well known correlation between the anemia of renal disease and the degree of nitrogen retention, that during the first twenty hospital days when signs of increased blood destruction and impaired erythropoiesis were most prominent, the blood urea nitrogen concentration ranged from 25 to 34 mg per cent (average 30 mg per cent), whereas during the subsequent thirty days when erythropoiesis and hemolysis were normal the blood urea nitrogen did not exceed 20 mg per cent (average value 17.5 mg per cent). No relationship was observed between the hematologic status and the grade of hematuria and proteinuria, or fluctuations in the total circulating protein.

A final comment is warranted regarding the influence of transfusion therapy on other manifestations of nephritis in this case. No evidence can be adduced that the course of the arterial hypertension, which was one of gradual improvement, was in any way affected by these maneuvers. Hematuria and albuminuria persisted without remission throughout the period of study. The transient elevations of total urinary albumin excretion following each series of transfusions, whether involving the injection of whole blood, washed red cells or purified albumin, presumably reflect an increased renal blood flow and glomerular filtration attending this therapy. Similar increases in proteinuria following the administration of albumin in cases of nephritis have been described by Thorn et al.<sup>27</sup> The progressive improvement in renal function as measured by changes in body weight, indicating increasingly effective water and sodium clearance, is readily attributable to the natural course of this patient's disease, rather than to variations in the degree of anemia. Thus, there was less water retention on the twenty-fifth hospital day, when his body weight was 69 kilograms, his blood volume 3650 cc and hematocrit 27.1, than on the fourteenth day when his body weight was 73 kilograms, blood volume 3960 cc and hematocrit 30.8. Similar conclusions obtain with respect to the observed reduction, between the eighteenth and twenty-first days, in the blood urea nitrogen concentration, these data being obtained in a patient whose renal decompensation was never severe, and whose clinical course was entirely consistent with one of progressive spontaneous improvement.

#### SUMMARY AND CONCLUSIONS

1. A 27 year old patient with an initial episode of acute glomerulonephritis was observed over a fifty day period, studies being directed primarily in an attempt to define the mechanisms responsible for a rapidly developing anemia. Hematologic data, including serial blood volume measurements and selective agglutination counts were obtained before and after the introduction of massive transfusion therapy.

2. The administration of group-O whole blood containing incompatible anti-A isoagglutinins in the first series of transfusions failed to improve the anemia but initiated a sustained reticulocyte response. Following this therapy there was evidence of increased blood destruction involving both the recipient's and the normal donor erythrocytes.

3. Data obtained following a second series of transfusions employing plasma-free group-O red cells, administered during a recovery phase when renal function had improved, indicated that blood destruction had largely abated and that hemopoietic activity was normal.

4. Two factors of undetermined origin are believed to have been implicated in the pathogenesis of anemia in this case: one, the occurrence of abnormally rapid blood destruction, and the other, impairment of blood formation. Both phenomena were associated with the presence of nitrogen retention, despite which, however, a prompt erythropoietic response followed the transfusion of whole blood with quantitative replacement of patient's red cells with donor erythrocytes, suggesting that previous bone marrow inactivity was not attributable to "toxic suppression."

## ACKNOWLEDGMENT

The author is greatly indebted to Dr Richard V Ebert, formerly Major, M C , A U S , for his valuable assistance in the planning and conduct of this experimental case study

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## STUDIES ON THE DESTRUCTION OF RED BLOOD CELLS \* IV

THEMAL INJURY ACTION OF HEAT IN CAUSING INCREASED SPHEROIDICITY, OSMOTIC AND MECHANICAL FRAGILITIES AND HEMOLYSIS OF ERYTHROCYTES, OBSERVATIONS ON THE MECHANISMS OF DESTRUCTION OF SUCH ERYTHROCYTES IN DOGS AND IN A PATIENT WITH A FATAL THERMAL BURN

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AND W B CASTLE, M D

IN A PREVIOUS communication<sup>1</sup> observations were reported on the changes in blood and urine and on the kidney complications occurring in 14 patients with moderate or severe thermal burns, 11 of whom showed hemoglobinuria. The spherocytosis and increased osmotic fragility of the red blood cells, observed in certain of these cases, were considered to result directly from the heating of the circulating blood. This paper reports detailed observations on the effect of heat on human erythrocytes and on the mechanism of destruction of heated dog red blood cells following their injection into the same animal. Finally, the characteristics of the red blood cells are reported in an additional case of a fatal thermal burn.

Previous investigations have established certain fundamental facts concerning the effect of heating red blood cells in the test tube and in the animal as a result of thermal burns. There is agreement<sup>2-7</sup> with the original observation of Schultze<sup>8</sup> that the heating of blood in vitro from human subjects, dogs, cats, and rabbits to temperatures of from approximately 52 to 65 C produces division and fragmentation of erythrocytes with the formation of spheroid forms of various sizes. In anesthetized animals, subcutaneous temperatures of from 51 to 65 C have been maintained for several minutes by scalding,<sup>9</sup> by igniting turpentine on the skin,<sup>10</sup> or by use of a hot iron.<sup>11</sup> Furthermore, it has been demonstrated,<sup>10 12 13</sup> especially by von Lesser,<sup>2</sup> that fragmented erythrocytes occurred in the blood stream in burned animals, in normal animals transfused with blood from a burned animal, and in normal animals transfused with blood heated in vitro. The abnormal erythrocytes disappeared rapidly<sup>2</sup> from the animal's circulation with the development of hemoglobinemia and hemoglobinuria. No hemolysins or agglutinins have been demonstrated in the plasma or serum of burned animals<sup>5</sup> or of burned patients.<sup>1</sup> An increase in the osmotic fragility of the red blood cells was noted in burned animals by Silberman<sup>3</sup> and in human blood heated in vitro by Isaacs, Brock, and Minot.<sup>7</sup> The latter investigators demonstrated that immature erythrocytes of both normal and pathologic human blood divided less readily than mature erythrocytes, when heated to 55 C. More recently hemoglobinemia, hemoglobinuria, fragmentation of erythrocytes, and increase in the osmotic fragility of red cells have been reported in a series of cases of human thermal burns by Brown<sup>14 15</sup> and in animals subjected to

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thermal injury by Moritz, Henriques, and Dutra<sup>16</sup> and by McLean, Moritz, and Roos<sup>17</sup> Hemoglobinemia and increased blood destruction have been observed as an early manifestation of severe burns in human subjects by Moore, Peacock, Blakely, and Cope<sup>18</sup> and in burned animals by Olson and Necheles<sup>19</sup>

## METHODS

The methods employed in this investigation have been described in previous communications as follows: determination of osmotic fragility of red blood cells<sup>1</sup>, determination of mechanical fragility of red blood cells<sup>20</sup>, estimation of the number of 'spherocytes' in a stained smear<sup>1</sup>, determination of hematocrit<sup>21</sup>, quantitative estimation of hemoglobin in plasma and urine<sup>22-24</sup>, measurement of pH of blood and urine by glass electrode method, measurement of methemoglobin and sulphhemoglobin<sup>2</sup>

## EFFECT OF HEAT ON ERYTHROCYTES

Changes in the shape, osmotic fragility, and volume of red cells were measured in 131 observations in defibrinated blood from 12 normal persons and from dogs. The blood was exposed to temperatures of from 25 to 70 C maintained for periods of from two minutes to one hour. All blood samples were heated, usually in 5 or 6 cc amounts, in soft glass test tubes of 13 mm diameter by 250 mm length. For larger volumes, pyrex Erlenmeyer flasks were employed. Each blood sample was first placed in a water bath at 37 C until this temperature was attained. Then the sample was immersed in a bath containing 30 liters of water which was agitated by a mechanical stirrer, and maintained at constant temperature with an accuracy of  $\pm 0.05$  C. A precision thermometer was introduced directly into the blood sample as it was heated and the temperature recorded every fifteen seconds, usually during gentle mixing with the thermometer. The blood sample was heated from 37 C to a particular temperature in approximately 2½ minutes, and maintained for a given time within  $\pm 0.05$  C at the required temperature. The sample was then removed from the bath and promptly cooled to 37 C in a water bath. The rates of heating and cooling were approximately equal. For the so-called "rapid heating" the water bath temperature was set 0.7 C above the final temperature desired for the blood sample. It required from one to two minutes in each observation to reach the desired temperature, at which time the blood was immediately removed and cooled as described above. Unheated and heated samples were compared with respect to hemolysis, osmotic fragility and shape of the red blood cells, hematocrit, pH, methemoglobin, sulphhemoglobin, and nonprotein nitrogen.

The changes produced in the red blood cells appeared in the following order: morphologic changes, apparent increase in volume, increase in osmotic and mechanical fragilities, and finally hemolysis in the serum or plasma. Temperatures up to 46 C for a period of one hour caused no demonstrable changes in the erythrocytes. At temperatures from 47 to 50 C changes in the red blood cells occurred depending on the temperature and duration of heating. At temperatures of from 51 to 65 C, changes always occurred even when the sample was subjected to "rapid heating." Changes occurred in dog red cells similar to those observed in human red cells. The various effects produced by heat are described separately below.

## A CHANGES IN MORPHOLOGY OF ERYTHROCYTES PRODUCED BY HEAT

The morphology of the red blood cells was studied in wet preparations made by diluting defibrinated blood with serum, physiologic saline, or Gower's solution and introducing one drop into a standard blood counting chamber or onto a glass slide, covering it with a glass coverslip and sealing with vaseline. In fixed preparations stained with Wright's stain, the red cells were examined for changes in size and shape, including the presence of "spherocytes" and "target" forms. The diameter of the red blood cells before and after heating was measured in stained preparations by the Price-Jones<sup>26</sup> method. Because of the bizarre forms produced by heating, these measurements serve only as an approximation.

TABLE 1—Effect on Morphology and Osmotic Fragility of Red Blood Cells Resulting from Heating Normal Human Defibrinated Blood at 48.6 C for Increasing Periods of Time (see Figure 1)

Duration of heating at 48.6 C	Apparent increase in hematocrit	Hemolysis after heating	Osmotic fragility of red blood cells Hemolysis, %								Morphologic observations on red blood cells						
											Segmented forms	Spherocytes	Microcytes	Mean Corpuscular Diameter,	Standard Deviation from Mean Diameter	Coefficient of Variation of Diameters	
			1	2.5	5	10	50	75	95								
Tonicity as NaCl Gm %																	
	%	%								%	%	%	microns	microns	%		
Unheated control	0	0	40	40	39	37	35	33	29	0	1	0	7.3	0.67	9.2		
Rapid heating	+0.6	0.4	40	40	39	37	34	32	23	0.5	0.2	0	7.3	0.52	7.1		
2 Minutes	+3.2	0	41	40	40	38	36	34	29	1	0.4	0	*	*	*		
5 Minutes	+5.8	0.4	40	40	39	37	35	33	30	5	1	0.6	*	*	*		
10 Minutes	+4.3	0.4	56	42	40	38	35	33	28	12	1.5	3	*	*	*		
30 Minutes	+6.7	0.7	79	76	68	59	40	36	29	4†	8†	*	5.5	1.0	18		
60 Minutes	+10.0	0.4	82	78	76	63	44	40	30	*	Many†	*	5.4	0.82	15		

\* Quantitative measurement was not possible

† Rough approximation

At temperatures of from 48.6 to 49.6 C, it was possible to observe the slow progression of the morphologic changes in the erythrocytes. A typical experiment conducted with heating of defibrinated blood at a constant temperature of 48.6 C for 2, 5, 10, 30, and 60 minutes, respectively, is illustrated in table 1 and figure 1. The first discernible change produced by heat was the appearance of small bud-like protrusions on an occasional erythrocyte. In the next recognizable change, many of the erythrocytes showed single or multiple buds usually connected by a broad base or a filament. When completely disconnected, the new elements formed small rounded or elongated structures containing various amounts of hemoglobin. At this stage, as illustrated in figure 1B and table 1 (2 and 5 minutes), there was no change in osmotic fragility of the red blood cells but apparent increases in hematocrit of 3.2 to 5.9 per cent, respectively. It is possible that the increase in hematocrit



in this and subsequent experiments did not represent a true swelling of the erythrocytes, but rather an inability to pack the malformed erythrocytes by centrifugation at 3000 r p m. This apparent increase in hematocrit occurred simultaneously with morphologic alteration of the erythrocytes, increased slightly with their progres

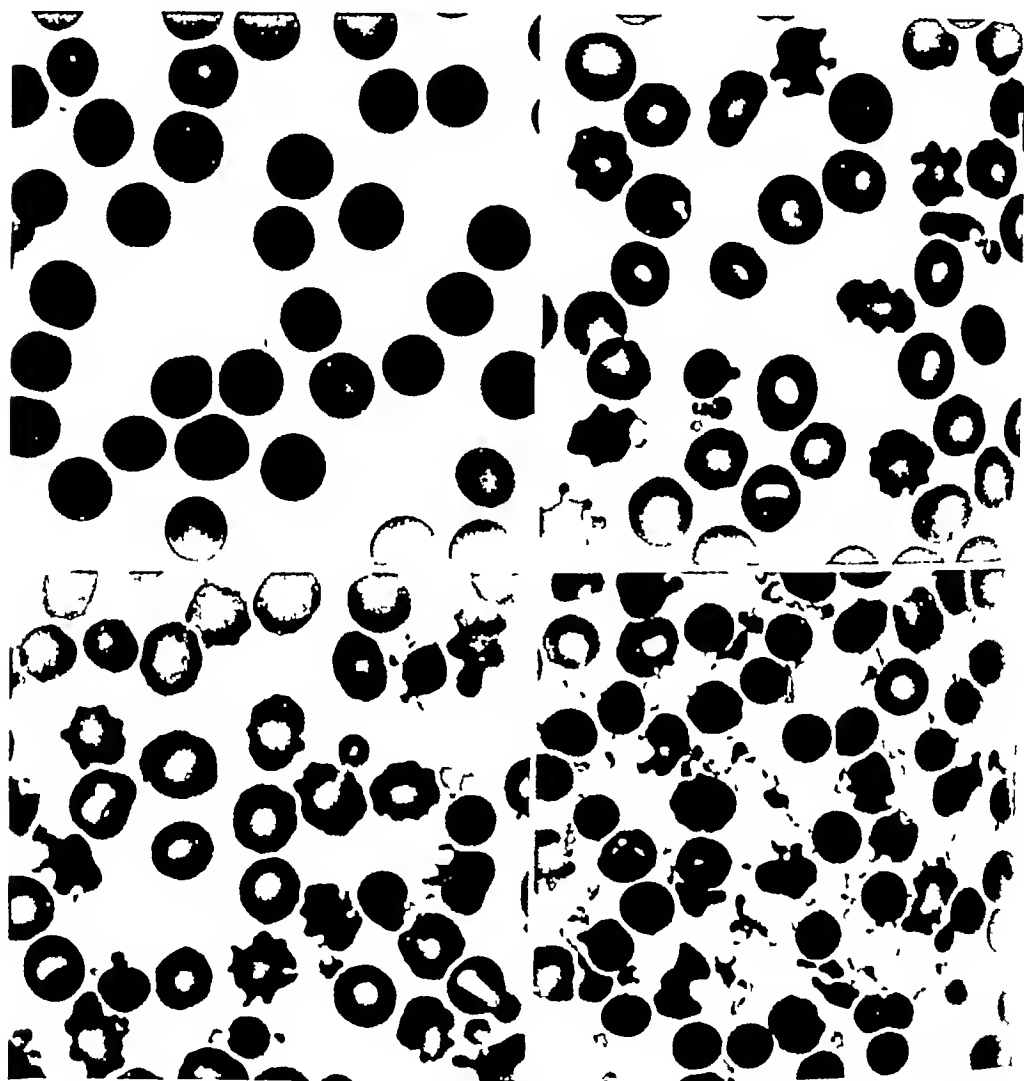


FIG. 1. EFFECT OF HEAT ON MORPHOLOGY OF RED BLOOD CELLS

A, upper left, unheated human defibrinated blood. Other samples heated at a temperature of 48.6°C, B, upper right, for 5 minutes, C, lower left, for 10 minutes, D, lower right, for 30 minutes. Stained blood films  $\times 1000$  (See table 1).

sive alteration, and thereafter did not vary in parallel with the increase in osmotic fragility. This phenomenon was not investigated further.

The first increase in osmotic fragility occurred after still further fragmentation of erythrocytes and coincidentally with the appearance of significant numbers of densely staining red cells of various sizes which appeared spheroid in shape in wet preparations and densely stained in fixed smears, as may be seen in figure 1C and table 1 (10 minutes). Continued fragmentation, as illustrated in figure 1D, resulted in the conversion of the majority of erythrocytes to spheroid cells, occasional

"ghosts," and many polymorphic fragments varying in size from 1-2 microns down to innumerable minute particles approximately the size of bacteria showing active Brownian movement in wet preparation. Microscopic examination of the erythrocytes in a wet preparation did not reveal Heinz-Ehrlich bodies<sup>27, 28</sup>

#### B CHANGES IN OSMOTIC FRAGILITY OF ERYTHROCYTES PRODUCED BY HEAT

Coincident with the continued fragmentation of erythrocytes, the osmotic fragility became significantly increased. The production of spheroid erythrocytes was evident from inspection of wet preparations and from the decrease in diameter and dense staining of red blood cells in stained preparations. As shown in table 1 (30 and 60 minutes), the increased osmotic fragility was also associated with a decrease in the mean diameter of the erythrocytes and an increase in the coefficient of variation of the diameters. It should be emphasized that progressive fragmentation of the erythrocytes occurred without significant loss of hemoglobin unless the osmotic fragility of a portion of the population had increased to such an extent that hemolysis occurred in concentrations of sodium chloride of from 0.85 to 1.0 grams per cent. Thus, when the osmotic fragility was normal, the free hemoglobin in the plasma was less than 1 per cent of that contained by the cells of the sample, this was also observed in many instances when both fragmentation and fragility were greatly increased, as shown in table 1 and in table 2. However, with sufficient heating, the osmotic fragility of the red cells could be so increased as to produce marked hemolysis of cells in serum or plasma. Thus, "rapid heating" at temperatures of from 55 to 60 C produced up to 5 per cent hemolysis, at temperatures of from 62 to 65 C from 22.5 to 100 per cent hemolysis.

In contrast to the morphologic changes in erythrocytes which were heterogeneous and difficult to evaluate quantitatively, the changes in osmotic fragility produced by heating were definitive and readily measured, as shown in figure 2. The change in osmotic fragility caused by a particular temperature and period of heating was remarkably reproducible for blood samples obtained at the same time or at different times from normal persons. When different blood samples were heated for a fixed period of time, a critical temperature was found, above which an increase of from only 1.0 to 1.6 C produced a phase of rapidly increasing osmotic fragility, as illustrated in figure 2 and table 2. Below this critical temperature range, the osmotic fragility was always found to be normal. For example, rapidly increasing osmotic fragility values occurred for "rapid heating" between 50.6 and 51.6 C, and for a period of sixty minutes of heating between approximately 47 and 48.6 C. Moderate and similar increases in osmotic fragility were produced by the following temperatures and periods of heating: at approximately 50.8 C by "rapid heating," and at approximately 49.2, 49, 48.6, 48.4, and 48 C by 2, 5, 10, 30, and 60 minutes heating, respectively. In order to illustrate the reciprocal relation between the effects of temperature and the duration of heating sufficient to produce a given increase in osmotic fragility, these data were plotted in two ways in figure 3. The approximately straight line function between the reciprocal of the absolute temperature and the logarithm of the time (equivalent to the rate), to be seen in figure 3B, suggests the conformity of enzyme reactions or chains of reactions with Arrhenius law.

TABLE 2.—Summary of Effect on Osmotic Fragility and Hemolysis of Red Blood Cells Resulting from Heating Normal Human Deformed Blood at Various Temperatures for Increasing Periods of Time

Tem- pera- ture of blood	Rapid heating				Heating for 2 minutes				Heating for 5 minutes				Heating for 10 minutes				Heating for 30 minutes				Heating for 60 minutes				
	Osmotic fragility RBC Hemolysis, %				Hemolysis after heating				Osmotic fragility RBC Hemolysis, %				Hemolysis after heating				Osmotic fragility RBC Hemolysis, %				Hemolysis after heating				
	Tonicity as NaCl Gm %				%				Tonicity as NaCl Gm %				%				Tonicity as NaCl Gm %				%				
	1	5	10	50	75	1	5	10	50	75	1	5	10	50	75	1	5	10	50	75	1	5	10	50	75
°C																									
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60 0																									
62 0																									
63 0																									
70 0																									

Control observations* on unheated blood											
Osmotic fragility of RBC Hemolysis, %											
Tonicity as NaCl Gm %											
1   5   10   50   75											
Maximum Minimum Average											
0 44	0 40	0 40	0 36	0 34	0 39	0 36	0 31	0 30	0 33	0 11	0 39
*20 observations on 3, three on 1, three on 2, three on 4, three on 5, three on 6, three on 7, three on 8, three on 9, three on 10, three on 11, three on 12, three on 13, three on 14, three on 15, three on 16, three on 17, three on 18, three on 19, three on 20.											

Control observations\* on unheated blood

Osmotic fragility of RBC Hemolysis, %

Tonicity as NaCl Gm %

1 5 10 50 75

Maximum Minimum Average

0.44 0.40 0.40 0.36 0.34 0.31 0.30 0.28 0.26 0.24 0.22 0.20 0.18 0.16 0.14 0.12 0.10 0.08 0.06 0.04 0.02 0.01

\*20 observations on 3 different normal test

rhénus' law<sup>29</sup> No attempt was made to interpret these data further. Henriques<sup>30</sup> has made a mathematical analysis of experimental time temperature relationships for thresholds of epidermal injury.

Observations were made of the changes in morphology and osmotic fragility produced by the heating of blood samples containing abnormally spheroid red cells from patients with chronic hypochromic anemia and sickle cell anemia, respectively. The results are summarized in table 3. In each instance, experimental conditions were arranged so that the heating of the blood sample increased the osmotic fragility of the red cells to approximately the same final value. The osmotic fragility of the red cells from the patient with congenital hemolytic jaundice was,

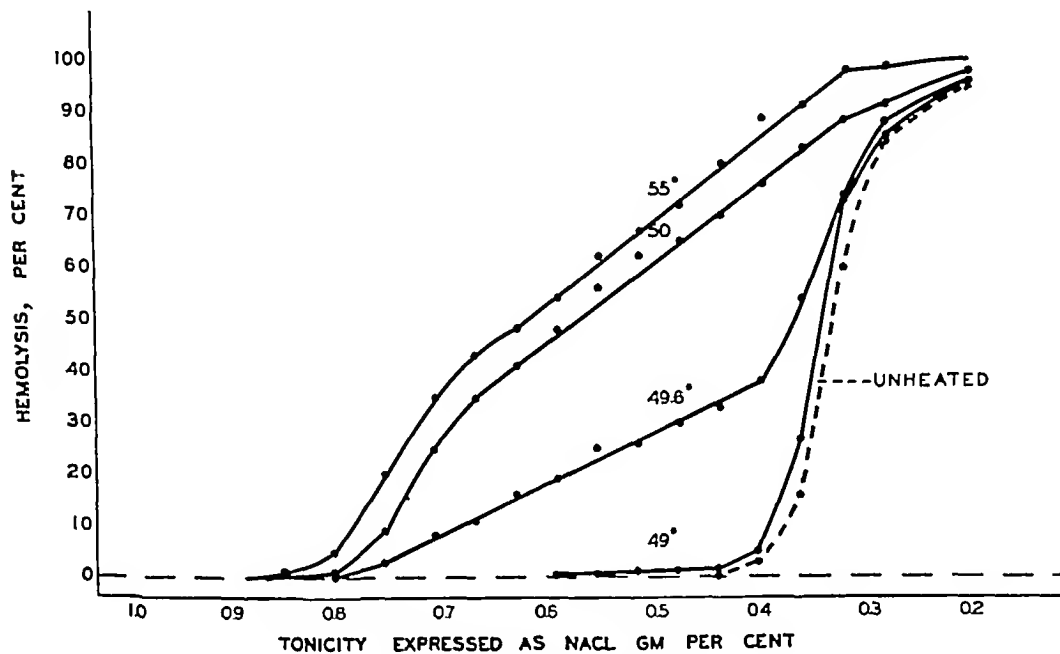


FIG. 2. OSMOTIC FRAGILITY OF RED BLOOD CELLS OF SAMPLES OF HEATED NORMAL HUMAN DEBRINATED BLOOD

The individual curves represent effects of heating for 2 minutes at temperatures of 49, 49.6, 50, and 55 C respectively, as indicated.

of course, already increased, and, before heating, stained films showed 3 per cent of 'spherocytes.' With heating, only a moderate degree of fragmentation and of decrease in mean corpuscular diameter was required to produce the observed increase in osmotic fragility. On the contrary, for the relatively discoid 'flat,' or 'target' cells with initially decreased osmotic fragility, the given degree of osmotic fragility was reached only after considerable morphologic change had appeared, indicated by marked decrease in mean corpuscular diameter and increase in the coefficient of variation of the erythrocyte diameters. Paradoxically, the temperature required to produce the given degree of morphologic change was greater for the spheroid than for the discoid cells. Thus, blood from the patient with congenital hemolytic jaundice required rapid heating to 52 C, that from the patient with sickle cell anemia to 50.6 C, in order to produce the same final value for osmotic fragility.

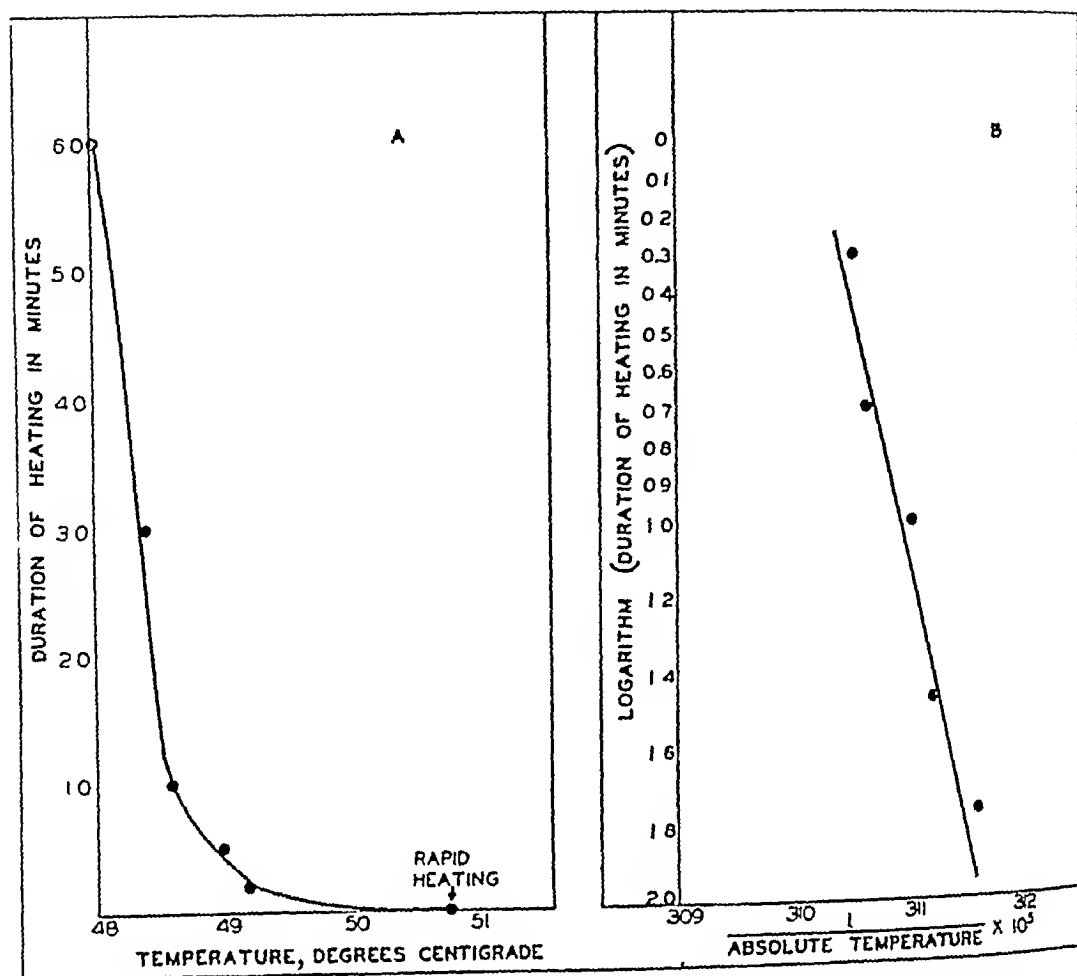


FIG 3 RELATION BETWEEN TEMPERATURE AND DURATION OF HEATING OF NORMAL HUMAN DEFIBRINATED BLOOD WHICH CAUSED APPROXIMATELY THE SAME FINAL DEGREE OF INCREASED OSMOTIC FRAGILITY

A (left) Duration of heating plotted against temperature B (right) The same data plotted as the logarithm of time (equivalent to  $\log \frac{1}{\text{rate}}$ ) against the reciprocal of the absolute temperature

The increase in osmotic fragility that was selected as a basis of comparison was as follows

Hemolysis, %	Tonicity expressed as NaCl, Gm %
1	0.74
2.5	0.68
5	0.55
10	0.43
50	0.34
75	0.30
95	0.4

The tonicities expressed here represent the average of several determinations or extrapolations of data falling near the value given

### C CHANGES PRODUCED BY HEAT AS A PROPERTY OF THE ERYTHROCYTE AND NOT OF THE MEDIUM

The changes in red blood cells produced by heat were investigated in order to determine whether the effects were reversible and whether inherent in the erythrocyte or dependent upon the presence of plasma or serum. A sample of normal de-

TABLE 3 — Comparison of Morphologic Changes in Red Blood Cells in which Heating Produced a Similar Osmotic Fragility of Blood Samples Containing Normal, Spherocytic, or Hypochromic Erythrocytes

Diagnosis	Temperature (rapid heating)	Hemolysis	Osmotic fragility of red blood cells										Morphologic observations on red blood cells						
			Hemolysis, %										RBC	Hemoglobin	Hematocrit	M C V (Reticulocytes)	M C Diameter (Morphology)	Standard Deviation From Mean Diameter	Coefficient of Variation of Diameters
			TONICITY AS NaCl Gm %																
			1	2.5	5	10	50	75	95										
Normal	Unheated	%	41	40	39	38	35	34	30	4 86	Gm %	44.9	92	73 (Normal morphology)	microns	0.50	6.9		
	50 C	0.6	82	79	76	72	39	34	25	4 7					1.6	35			
Congenital hemolytic jaundice	Unheated		60	56	51	44	40	39	36	3 60	9.4	30.8	86	65 (spherocytes, 3%)	0.74	11			
	52 C	0.8	78	77	75	71	50	43	35					53	0.94	18			
Sickle cell anemia	Unheated		48	37	33	29	22	17	08	4 78	9.8	31.3	66	68 (rare sickled forms, target cells, 12%)	0.96	14			
	50 C	0.5	80	78	76	73	47	26	13					36 (rare sickled forms, target cells, 1%)	1.2	33			

fibrinated human blood was kept at a temperature of 53 C for a period of nine minutes, producing a marked increase in osmotic fragility. The heated blood and a sample of unheated blood were then centrifuged and the serums removed and interchanged in such amounts as to produce a 5 per cent suspension of heated cells in unheated serum, and the reverse. These two mixtures, together with unmanipulated samples of heated blood and unheated blood were then introduced into separate 250 cc tonometers which were closed and rotated slowly for two hours in an incubator at 37.5 C. There was no change in the abnormally increased osmotic fragility of the heated red cells produced by the fresh unheated serum and no significant hemolysis or increase in the osmotic fragility of the unheated cells suspended in the

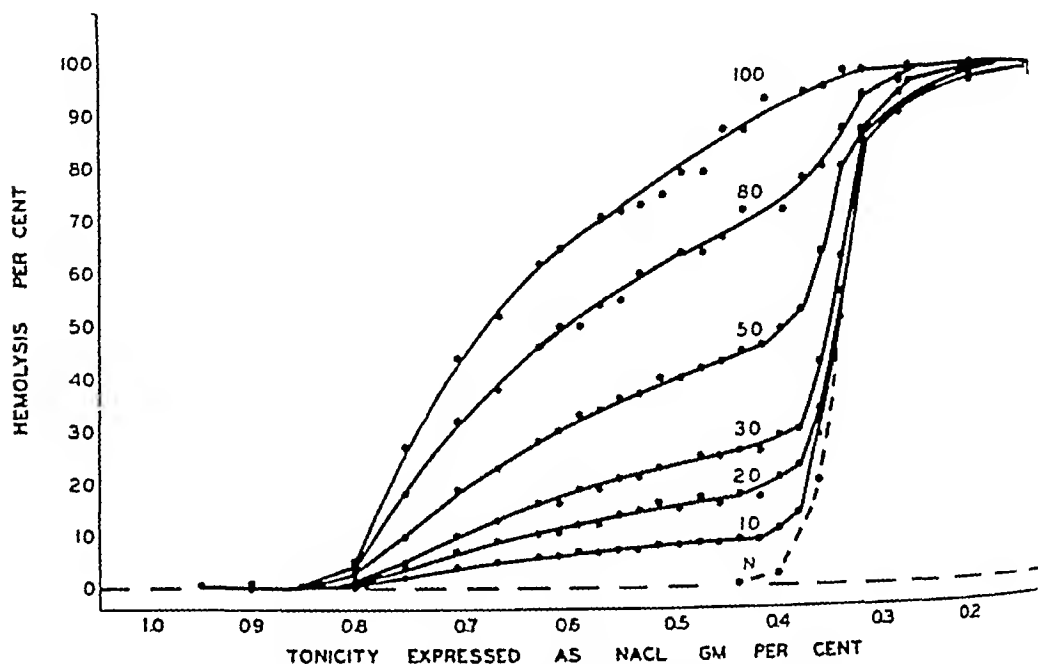


FIG 4 Osmotic Fragility of Red Blood Cells in Mixtures of Heated and Unheated Normal Human Defibrinated Blood

N, unheated sample, 100 (per cent by volume of blood), after rapid heating to 55 C, 10-80, mixtures of varying proportions of the heated sample (10-80 per cent by volume) with the unheated sample

heated serum. When mixtures of from 10 to 80 per cent by volume of heated blood exhibiting increased osmotic fragility were made with normal blood, the resulting osmotic fragility curves showed the values to be expected from such a mixed population (fig 4).

In another experiment, a sample of plasma (containing sodium citrate 500 mg per 100 cc) and of serum were heated at 56 C for two minutes and for thirty minutes, respectively. The precipitated fibrin was then removed from the heated plasma by centrifugation. Five per cent suspensions of unheated red cells were then made in each and were incubated in tonometers as described above, for the same time as unheated samples of whole citrated and defibrinated blood. No significant changes occurred in the osmotic fragility or degree of hemolysis of any of the six samples.

The effect of different mediums on the increases in osmotic fragility of red cells

caused by heat was investigated. Whole blood containing heparin, 150 mg per 100 cc, defibrinated blood, and red cells from defibrinated blood washed and resuspended in isotonic salt solution, were heated for ten minutes in separate test tubes under identical conditions. At 48.8°C, a moderate and similar increase in osmotic fragility was produced in each. At 49.6°C, however, the red cells in isotonic saline showed a somewhat greater increase in fragility and a significantly greater degree of hemolysis, i.e., 16 per cent, compared to 2 and 1 per cent, respectively, for the other two samples. Similarly, washed red cells suspended in isotonic saline, when heated up to 53°C in three minutes, showed a greater increase in osmotic fragility than did the red cells from defibrinated blood that was treated similarly. Hemolysis of the red cells in isotonic saline was 34 per cent, but only 2 per cent in defibrinated blood. However, when the red cells of the heated defibrinated blood sample were washed and resuspended in isotonic saline, they exhibited the same increase in osmotic fragility as those originally heated in suspension in saline. In contrast to heparin, sodium citrate, 250 mg per 100 cc, when used as anticoagulant for whole blood or when added to defibrinated blood caused a significantly greater increase in fragility and in hemolysis when the samples were heated than was observed with heated defibrinated blood. The possible effect of other salts was not investigated.

The increase in osmotic fragility of the red cells was not accompanied by any significant change in the nonprotein nitrogen concentration of heated defibrinated blood or of erythrocytes washed and suspended in isotonic sodium chloride solution. There was no production of methemoglobin or sulphemoglobin. The hydrogen ion concentration of the suspension of red cells, as measured by the glass electrode, was not materially changed for heated whole blood containing heparin or sodium citrate 250 mg per 100 cc, for defibrinated blood, or for erythrocytes washed and suspended in isotonic saline.

#### D. EQUILIBRIUM VOLUMES OF HEATED ERYTHROCYTES

The equilibrium volume of erythrocytes (hematocrit) from samples of normal and heated human defibrinated blood was tested by a modification of the method of Castle and Daland<sup>31</sup> who emphasize the fact that differences in osmotic fragility of different types of erythrocytes are not explained by differences in strictly osmotic properties, but rather by differences in the shape of red cells. The purpose of these experiments was to determine what alterations, if any, were produced by heat in the permeability of the membrane of the red cell or in the osmotic activity of its contents. Because no striking changes in the hematocrit of samples of defibrinated blood were noted to occur as a result of heat, such effects appeared to be minimal.

Aliquot samples of human defibrinated blood were heated rapidly to 49.6 and 50.6°C, thus producing a slight and an extreme increase in osmotic fragility, respectively, as shown in table 4. Then 1.0 cc amounts of these two samples and of an unheated aliquot were mixed with 1.0 cc amounts of solutions of sodium chloride ranging in concentration from 0.51 to 1.7 grams per cent. After mixing, the hematocrit of each sample was determined. Then the percentage difference between that value and the value obtained when the sample was mixed with sodium chloride solution 0.85 grams per cent was computed. The tonicities of the mixtures of serum



and salt solution were calculated, assuming the serum tonicity to be isotonic with 0.85 grams per cent sodium chloride solution. In the experiment shown in table 4, the original red cell volume was 48, the serum volume 52 per cent. The hematocrit readings were probably only approximate in accuracy because of the polymorphic nature of the heated red cells. Moreover, because of their increased osmotic fragility a significant portion of the heated red blood cells hemolyzed in the hypotonic mixtures of sodium chloride. In the hypertonic solutions, however, hemolysis did

TABLE 4—*Change in Equilibrium Volume of Unheated and Heated Normal Defibrinated Human Blood Mixed With an Equal Volume of Hypotonic or Hypertonic Solutions of Sodium Chloride and Compared to the Hematocrit in Isotonic Sodium Chloride*

Concentration of solution of NaCl, Gm % mixed with blood	Calculated tonicity* of mixture of serum and NaCl solution, expressed as NaCl, Gm %	Control unheated		Rapid Heating to 49.6 C		Rapid Heating to 50.6 C	
		Hematocrit	Change in hematocrit	Hematocrit	Change in hematocrit	Hematocrit	Change in hematocrit
		%	%	%	%	%	%
1.7	1.41	19.0	-20.1	19.0	-19.5	19.1	-20.4
1.36	1.19	20.2	-15.8	20.1	-14.8	21.0	-12.5
1.19	1.08	21.0	-12.5	21.7	-8.0	21.7	-9.6
1.02	0.96	22.0	-8.3	22.3	-5.5	22.9	-4.6
0.85 (isotonic)	0.85	24.0	0	23.6	0	24.0	0
0.68	0.74	25.1	+4.6	25.6	+6.8	Hemolysis 2+	—
0.51	0.63	27.2	+13.3	Hemolysis ± Hemolysis +	—	Hemolysis 3+	—
Tonicity as NaCl Gm %							
Osmotic fragility,	1	41		79		81	
Hemolysis, %	5	39		62		77	
	10	37		44		76	
	50	33		34		59	
	75	32		26		39	

\* Serum was considered to have a tonicity equivalent to NaCl, 0.85 Gm per cent

not occur and the percentage decrease in hematocrit was roughly the same for the heated and unheated sample. This indicates that, as with red cells of naturally occurring different osmotic fragilities, the strictly osmotic behavior (percentage change in equilibrium volume with change in tonicity of suspension medium) of the heated red cells did not differ significantly from that of the normal red cells.

#### B. EFFECT ON HEATED ERYTHROCYTES OF SUBSEQUENT INCUBATION AT 37.5 C

Samples of normal human and dog defibrinated blood were so heated as to produce a moderate increase in osmotic fragility. Then 6 cc amounts of heated and un-

heated samples of each were incubated in 250 cc tonometers with slow rotation at 37.5 C for periods of 4, 8, or 10 hours. A significant increase occurred in the osmotic fragility of the heated, compared to the unheated, samples of dog blood. The increases in the osmotic fragility of the heated human red cells were minimal even after ten hours, as were those of the unheated controls. This corresponds with previous observations<sup>32, 33</sup> upon the sterile incubation of normal and pathologic human blood and indicates, apparently, an increased susceptibility of the heated dog red cells to such incubation.

#### F CHANGES IN MECHANICAL FRAGILITY OF ERYTHROCYTES PRODUCED BY HEAT

The mechanical fragility of samples of both heated and unheated human and dog blood was determined. For example, samples of defibrinated dog blood were subjected to "rapid heating" at 49.8, 52.8, and 54 C. Immediately thereafter, the hematocrit and osmotic fragility of each of the heated bloods were determined. The hematocrits of the unheated samples were adjusted by removal of serum to equal those of the samples exposed to the highest temperature. The hematocrits of the other heated samples were not adjusted in each experiment. The mechanical fragility of a given sample was taken as the percentage of its hemoglobin liberated by standardized trauma from glass beads rolling in a rotating tonometer.<sup>20</sup>

Heating of blood that was just insufficient to cause increase in osmotic fragility had no detectable effect on mechanical fragility. However, the effect of sufficient heat was to produce progressive increases in both the osmotic and mechanical fragilities of the erythrocytes. The increase in mechanical fragility was roughly proportional to the increase in osmotic fragility. Thus, for example, as may be seen from figure 5, rapid heating to temperatures of 52.8 and 54 C caused the mechanical fragility of a sample of dog blood to increase from a control value of 7.0 per cent to 20.2 and 31.5 per cent, respectively. A temperature of 52.2 C caused the mechanical fragility of a sample of human blood to increase from a control value of 3.9 per cent to 16.3 per cent (no figure shown).

Continuous trauma of heated defibrinated human or dog blood for periods of from three to eight hours apparently did not destroy selectively those red cells that showed the greatest increase in osmotic fragility. This was evidenced by observation of the curve of osmotic fragility at frequent intervals while hemolysis from trauma was progressing. With human blood there was no evident change in shape of the osmotic fragility curve to indicate alteration of all or selective destruction of any particular portion of the cell population. On the contrary, samples of defibrinated dog blood that were heated sufficiently to produce increased osmotic and mechanical fragility of the red cells when subjected to continuous trauma for fifty minutes or at intervals for three hours, showed a significant uniform and progressive increase in osmotic fragility as the hemolysis from trauma increased. It was assumed, as a hypothesis without further investigation, that the progressive increase in osmotic fragility resulting from the continuing trauma of heated dog blood might be the result of an increase in the degree of fragmentation of the red cells already initiated by heat.

## EFFECT OF INJECTION OF HEATED ERYTHROCYTES INTO DOGS

In previous observations<sup>1</sup> on patients with thermal burns, hemoglobinemia and hemoglobinuria were found. It is clear from the preceding experiments that, in blood samples heated *in vitro*, striking increases in both osmotic and mechanical fragilities of the red cells were demonstrated. In order to determine whether such red cells would be readily destroyed *in vivo*, blood was removed from normal dogs, heated, and injected into the same animal.

In the several experiments, 8 healthy dogs in the fasting state, weighing from 12 to 18 Kg, were bled, using sterile precautions, from the jugular or femoral veins

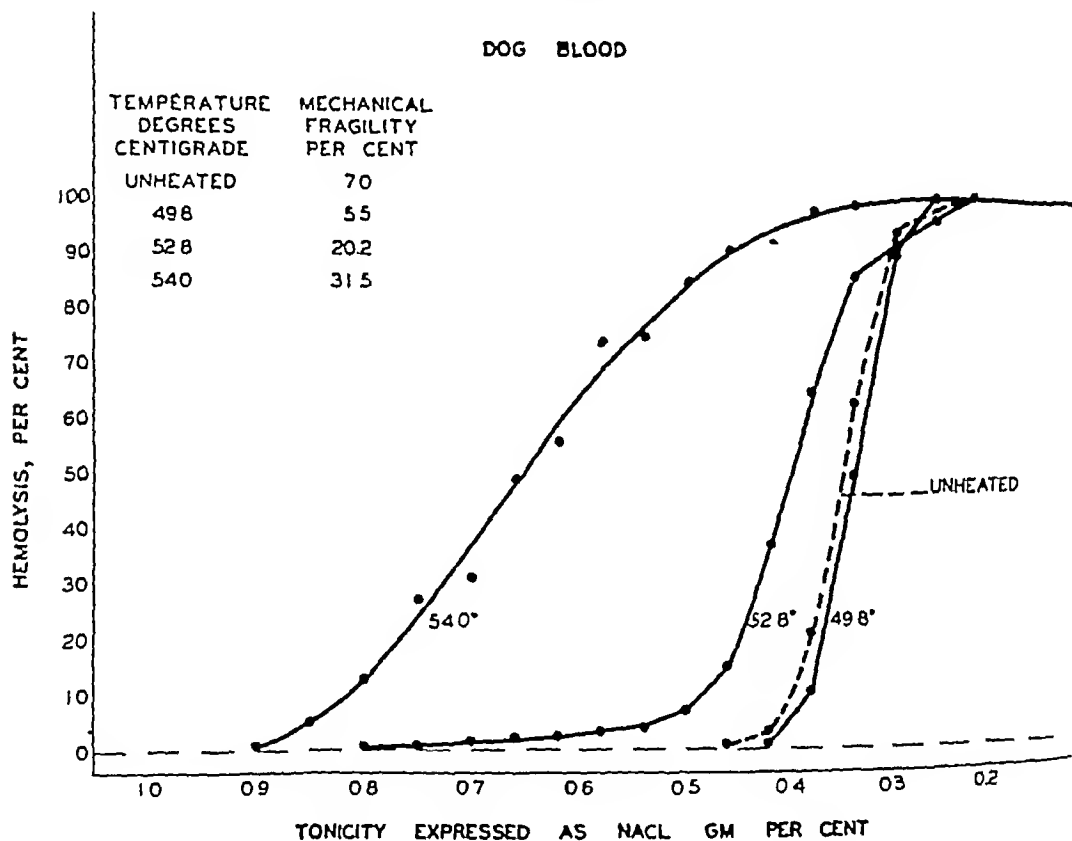


FIG 5 OSMOTIC AND MECHANICAL FRAGILITIES OF RED BLOOD CELLS OF DOGS DEFIBRINATED BLOOD AFTER 'RAPID HEATING' TO 49.8, 52.8, AND 54.0 C, RESPECTIVELY, AS INDICATED

from  $\frac{1}{6}$  to  $\frac{1}{3}$  of their blood volume, as estimated from their body weight.<sup>21</sup> The blood was defibrinated or citrated as drawn. In Dogs 1 and 5, ether anesthesia with an open cone was used briefly during the venesection. Immediately after the bleeding, an equal volume of isotonic sodium chloride solution containing 40 cc of molar sodium lactate was injected intravenously. The blood sample removed was treated as described below and later injected intravenously into the same animal. Thereafter, the animal was given food and water *ad libitum*. Samples of venous blood were taken at frequent intervals, before and after the injection, with precautions to prevent hemolysis of the samples by trauma.<sup>24</sup> On these samples were determined especially the morphology and osmotic fragility of the erythrocytes, the plasma hemoglobin<sup>24</sup> and the hematocrit. In experiments on Dogs 6, 7,

and 8 the mechanical fragility of the red cells was also determined. All urine samples were collected. The rectal temperature was recorded at frequent intervals.

*Dog 1 received his own red cells which were heated in serum and then washed and resuspended in saline, as described below, in order to eliminate any potential hemolytic or toxic factor contained in the heated serum. From Dog 1, weighing 12 Kg, approximately  $\frac{1}{3}$  of the calculated blood volume, 250 cc, was withdrawn, defibrinated, heated in a water bath at 54 C, during a period of twelve and one-half minutes required for the temperature of the blood to reach 52.2 C. The sample was then immediately cooled to body temperature, centrifuged, the serum discarded, and the red cells washed three times with 2 volumes of hypertonic (1.275 grams*

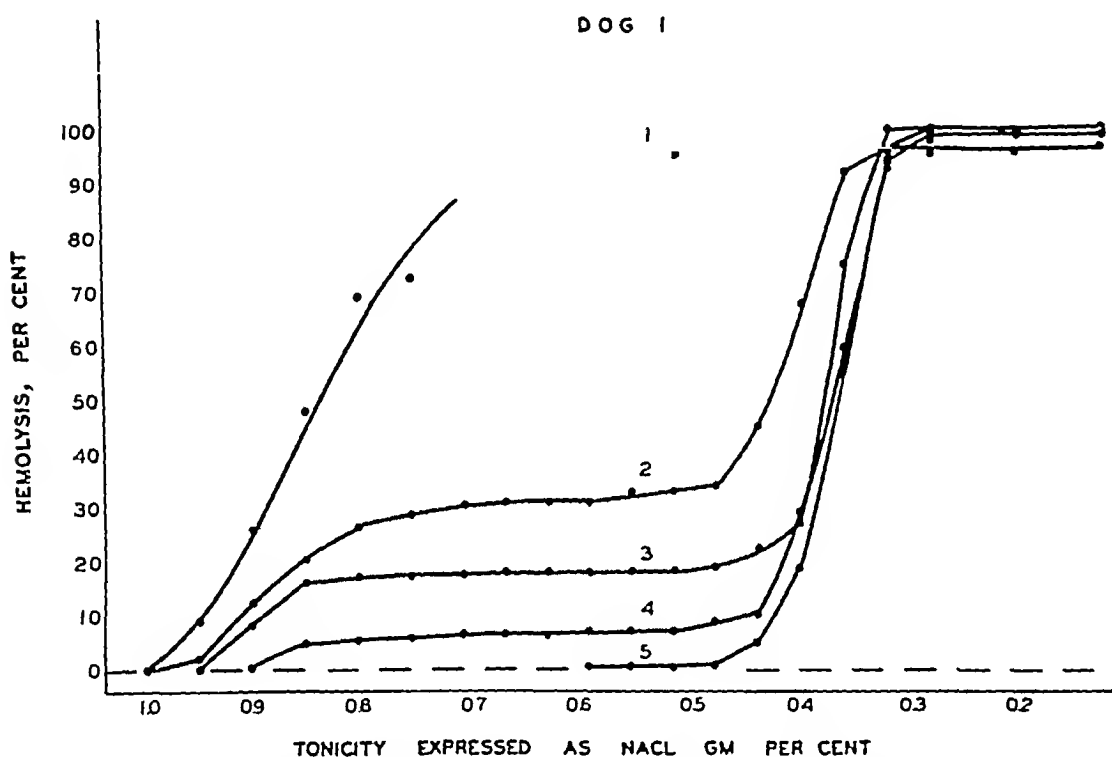


FIG. 6. DOG 1. EFFECT OF INTRAVENOUS INJECTION OF WASHED RED BLOOD CELLS FROM 250 CC HEATED DEFIBRINATED DOG'S BLOOD UPON OSMOTIC FRAGILITY OF CIRCULATING RED BLOOD CELLS.

Curve 1, Washed red cells before intravenous injection. Curve 2, immediately after injection. Curve 3, after 2 hours. Curve 4, after 7½ hours. Curve 5, after 41 hours. See also figure 7. Note that approximately 30 per cent of the red cell population was abnormal immediately after injection (curve 2). Subsequently the osmotically fragile red cells progressively disappeared.

per cent), sodium chloride solution. After the red cells were resuspended, at their original hematocrit, in the hypertonic saline, they exhibited an extreme increase in osmotic fragility as shown in curve 1 in figure 6. Immediately after the intravenous injection of the suspension, the animal's venous blood showed a mixed red blood cell population with approximately 30 per cent of cells of increased osmotic fragility and 70 per cent of normal cells, as indicated in curve 2 in figure 6. This type of fragility curve resembled that occurring in artificial mixtures of heated and unheated red blood cells, as shown in figure 4. The subsequent osmotic fragility curve showed a gradual disappearance of the osmotically fragile cells during eighteen hours after injection. Immediately after the injection of the heated red cells, although no free hemoglobin was detectable in the supernatant plasma, a small amount of hemoglobin was noted in the animal's plasma a day later. The maximum value for plasma hemoglobin was 1.6 grams per 100 cc of plasma.

transfer, in one or another form, of fetal red blood cells into the maternal circulation Javert<sup>14</sup> suggested that fetal red blood cells may enter the maternal circulation via placental thrombi in which he claims to have observed fetal red blood cells. This view could not be confirmed by Potter<sup>15</sup> in a study of 60 placentae which had thrombus-like lesions. Burnham<sup>16</sup> claimed that lack of or improper utilization of vitamin C produces placental lesions which would permit leakage of fetal red blood cells. Because of lack of evidence this view is generally not accepted.

In the first studies which demonstrated the role of the Rh factor in the pathogenesis of erythroblastosis fetalis, no attempt was made to describe the mechanism by which the fetal red blood cells, a large formed element, traverse the placenta. In the interval of 1941-1943, a concept was gradually developed which seemed to be compatible with the clinical and serologic findings.<sup>17</sup>

It is assumed that in every normal pregnancy minute quantities of fetal red blood cells, in one form or another, find their way into the maternal circulation in sufficient quantity to induce isoimmunization. According to this view, it is not necessary to assume the existence of gross placental lesions, which would have to recur with each successive pregnancy in which the fetus is Rh+. Another consideration is the fact discussed below, that only minute quantities of blood may suffice to immunize. The existence of gross placental lesions can also be excluded since the course of the pregnancy and the delivery in the vast majority of immunized mothers is entirely uneventful. Toxic symptoms are apt to occur only in some of the much smaller group of mothers of infants suffering from fetal hydrops and it is probable that these symptoms in the mother cannot be attributed to the effects of gross leakage.

From an immunologic viewpoint, it is safe to assume that minute quantities of fetal blood may suffice to induce isoimmunization. There are numerous examples in serologic literature to demonstrate that very small doses of soluble proteins, toxins, suspensions of bacteria or red blood cells administered to the experimental animal will result in antibody production. Perhaps the most outstanding example is sensitization of the guinea pig to anaphylaxis by the injection of, e.g.,  $10^{-6}$  cc. of horse serum (ca.  $7 \times 10^{-8}$  grams of protein).

In order to accumulate additional evidence with red blood cells as an antigen, the author injected a series of rabbits daily with 2 cc. of a 1:5000 suspension of citrated human blood. After seven injections there was a slight to moderate increase in the activity of the sera for human blood. The antibody response was more striking after a second series of injections (table 2).

It is of interest that one of the animals was comparatively resistant to the injections and that one of the remaining five animals produced anti-M agglutinins as well as species-specific antibodies. Measured in terms of sediment, the total volumes employed for the seven and fourteen injections were respectively, 0.0014 and 0.0028 cc. The corresponding values for an adult of 120 lbs (60 Kg) are 0.0336 and 0.0672 cc. respectively.

It is true that the above-mentioned experiment dealt with heteroimmunization and it seems desirable to determine whether or not the same principle applies also to isoimmunization of rabbits with minute quantities of rabbit blood of varying

antigenic constitution. However, there is already indirect evidence obtained from recent experiments on isoimmunization of Rh— donors with small quantities of Rh+ blood. This applies especially for the increase of antibodies already formed. From a practical viewpoint, one must consider that these experiments cannot simulate the conditions existing in pregnancy which are most favorable for antibody production, i.e., slow administration of the antigen over a long period. Certainly these conditions cannot be satisfied in either abortions, ectopic pregnancies, nor in the process of parturition.

By and large the effects of isoimmunization by the Rh factor are exerted on the fully, or almost fully developed fetus, or the newborn infant. Intra-uterine fetal death in the seventh or eighth month is never observed in the first born unless the mother has been immunized by previous transfusions of Rh+ blood. With increasing degrees of isoimmunization, fetal death may occur but scarcely before the sixth or seventh month. Remarkably enough, the presence of potent antibodies residual from the preceding pregnancy does not interfere with the process of fertilization, implantation, nor with the subsequent development and growth of the fertilized

TABLE 2 \*—*Immunization of Rabbits with Minute Quantities of Human Blood (Group O)*

Time of Test	Agglutination Titers of Five Immunized Rabbit Bloods Tested with Blood Group O				
	1	2	3	4	5
Before injections	5	10	5	5	5
After 7 injections	15	100	25	25	10
After 14 injections	100	300	25	600	100

\* After Levine<sup>17</sup>

ovum, even though red blood cells are already in the process of formation in the fourth week.<sup>20</sup> The Rh factor has been demonstrated in the blood of a 48 mm fetus by Stratton,<sup>21</sup> and a 17 cm fetus by Bornstein.<sup>22</sup> Diamond<sup>23</sup> demonstrated the Rh factor in the blood of three fetuses of 3 month development while Potter<sup>15</sup> found the factor present in fifteen out of seventeen fetuses weighing between 8 and 200 grams. There is reason to suspect that the more fundamental property of antigenicity and capacity to unite with antibodies may be inherent in the earlier fetus or even in the embryologic forerunners of red blood cells.

Although immunized Rh— mothers have a somewhat higher incidence of abortions and miscarriages,<sup>2-24</sup> there is little or no statistical proof to indicate that early fetal death is brought about by the action of passively transferred maternal antibodies. While metabolites are transferred early in the course of development, there is no proof that the early fetus receives maternal antibodies. Certainly, the fetus does not require antibodies at this early stage of its development. In any event, this subject and its possible relationship to early fetal death merits further investigation. Whether or not an early abortion followed by a curettage results in the transfer of fetal blood into the maternal circulation with subsequent isoimmunization<sup>15</sup> will be referred to below.

According to the author's concept, isoimmunization by the Rh factor does not

begin until after midpregnancy when certain structural changes occur which are favorable for the transfer into maternal sinuses of minute amounts of one form or another of fetal red blood cells.<sup>17</sup> As the placenta grows the blood vessels in the villi, at first centrally located, become larger and come to lie adjacent to the maternal sinuses. During this period the Langhans cells degenerate and only a thin membrane and one layer of syncytial cells separate the fetal and maternal circulations. The gradual thinning of the barrier is readily demonstrated in a comparative study of histologic sections of normal placentae of varying periods of development. The retention of Langhans cells in the placentae of erythroblastotic infants does not invalidate the view presented since these changes may be considered as secondary responses to the hemolytic process.

At the same time there is an ever-increasing surface area of fetal villi in contact with maternal sinuses. It has been calculated by Dodds<sup>25</sup> and Dees-Mattingly<sup>24</sup> that in the term placenta there are from 70-120 square feet of fetal villi exposed to maternal sinuses and the total length of these villi, if laid end to end, would measure 11 4 miles. Furthermore, one fourth or more of the fetal blood is outside of the fetus and in close contact with the maternal circulation. It is known that the circulation in the maternal sinuses is very sluggish and that the pressure is greater in the fetal circulation. Accordingly, there is ample opportunity even under physiologic conditions for the escape into the maternal sinuses of a minute number of red blood cells in one or another form. One may well speculate that the pressure in the fetal villi is not constant, and may be increased as a result of fetal movements which generally become active in the fifth month.

The mechanism of isoimmunization described is compatible with the clinical observation that once an Rh- mother delivers an erythroblastotic infant, the condition is apt to recur in all succeeding pregnancies in which the fetus is Rh+. Apparently, the isoimmunization is renewed even if subsequent pregnancies are spaced at intervals which are sufficiently long for the complete disappearance of antibodies, residual from the preceding pregnancy. Thus, the specific form of the anamnestic reaction is called into play in each successive pregnancy, and the determining factor is the presence of Rh+ fetal red blood cells in the maternal circulation during the course of the pregnancy.

Transplacental isoimmunization by the Rh factor depends on (1), a combination of an Rh- mother and Rh+ fetus and (2), genetic capacity to respond to the antigenic stimulus. It is assumed that the mother whose first Rh+ infant is affected produces antibodies with greater ease than the mother who has several normal Rh+ infants prior to the first affected infant. These two facts serve to determine the comparatively low incidence of erythroblastosis in spite of the fact that there are 13 per cent incompatible matings ( $85 \text{ per cent} \times 15 \text{ per cent} = 13 \text{ per cent}$ ). More than 50 per cent of these Rh+ fathers (about 58 per cent) are heterozygous and half of their offspring will be Rh-, like the mother. Another factor tending to reduce the incidence of erythroblastosis fetalis is the current tendency to small families. It is probable that every Rh- mother would produce an erythroblastotic infant provided that there were a sufficient number of pregnancies.

The same genetic factors determining the capacity to produce antibodies are

also operative in isoimmunization of voluntary donors by administration of Rh+ blood. Thus, Diamond and Wiener report successful isoimmunization in somewhat less than 50 per cent of the Rh- donors. Of 100 Rh- random individuals who were transfused indiscriminately, about 46 per cent produced antibodies for Rh. It is most significant that the antigenic response was independent of the number of transfusions, but determined rather by certain genetic properties. In contrast to the patient reported by Dacie and Mollison<sup>28</sup> who produced antibodies soon after the first transfusion is an Rh- patient studied by Levine who produced antibodies after a series of numerous transfusions.<sup>29</sup>

As pointed out by Levine, the occurrence of erythroblastosis fetalis in the first born with or without previous transfusions has considerable bearing on the mechanisms of transplacental isoimmunization. In both groups of cases, there is a selection of those Rh- women who produce antibodies readily, but of the two, the nontransfused group is obviously the more sensitive to the administration of Rh+ blood. The objection has been raised that many of these women had one or more abortions, probably premarital, which are not obvious from the usual history. This raises the question whether or not fetal red blood cells may be carried over into the maternal circulation as a result of either a spontaneous abortion or the subsequent curettage. However, this is not likely, particularly if there is considerable uterine bleeding which would tend to carry with it the fetal blood. In any event, an early pregnancy cannot supply the conditions favorable for isoimmunization, i.e., slow administration of the antigen over a long period. In a number of cases studied by the writer a history of abortion was denied. A more important consideration is the likelihood that many of these women had previously received an intramuscular injection of presumably Rh+ blood.<sup>31</sup> In the pre-vitamin K days, the intramuscular injection of blood was a common practice and more recently such histories were obtained, particularly if the indication was prophylaxis for either measles or poliomyelitis. Under such conditions the erythroblastosis fetalis in the first born is classified under the "transfused" group.

Erythroblastosis fetalis in the first born is a more common event in the transfused group and in many of these there is a long interval between the last transfusion and the first pregnancy. It is not likely that the antibodies produced as a result of one or more transfusions will be demonstrable for more than several years, but certainly not for five years, at least in the vast majority of the cases. Accordingly, it may be assumed that in many of these cases, no antibodies are present when the first pregnancy is started. The subsequent appearance of antibodies during the course of the pregnancy provides proof that fetal Rh+ blood cells must have passed the placental barrier in sufficient quantity to exert its antigenic effect.

The following case of erythroblastosis fetalis in the first born referred by Dr. Regina Beck of Richmond, Virginia is cited because antibodies, which were not demonstrable either early in the course of the pregnancy, i.e., the twelfth week or in the thirty-third week, were found in a specimen drawn in the thirty-eighth week (titer = 1:64). This Rh- patient was transfused six times in 1941 for gastroenteritis, five years prior to her first pregnancy. It is significant that in the specimen drawn seven weeks prior to the expected date of confinement (July 17, 1947)



the serum failed to sensitize Rh+ cells so that the precipitin reaction of Coombs, Mourant, and Race<sup>32</sup> was completely negative. In view of her history, the patient's serum was titrated and as was to be expected, the existence of a prozone in the lower dilutions was excluded.<sup>33</sup> Assuming a two or three months' preparatory period, one may speculate that fetal red cells began their passage between the twenty-sixth and thirtieth week of the pregnancy. Since there were six transfusions, of which probably five were with Rh+ blood, the patient must be considered as relatively resistant to the production of antibodies.

Curiously enough there are remarkably few cases in which there are sufficient data useful for this analysis. As a rule these patients do not submit to the test until late in the course of the pregnancy when antibodies are already demonstrable and their origin, whether residual or newly formed, cannot be determined. However, indirect evidence may be obtained in an analysis of the interval between the last transfusion and the first pregnancy with an Rh positive fetus. In a combined series of 52 cases of erythroblastosis fetalis in the first full term Rh+ infants, the average interval was six years and as mentioned above it is safe to assume that (1) the antibodies which may have been present earlier will have disappeared in the six years' interval and (2) that the appearance of antibodies responsible for erythroblastosis fetalis was the result of intra-uterine passage of fetal blood.\*

Of the 52 cases, 10 had previous histories of abortions or ectopic pregnancies and 9 of the 10 required blood transfusions. The average interval between the transfusion and the first pregnancy resulting in an erythroblastotic infant was 3.7 years. The shorter interval does not indicate that the abortions resulted in isoimmunization. In women of child-bearing age, one cannot expect to find long intervals between pregnancies. Excluding this series, the interval in the remaining 42 cases between the transfusions and the first pregnancy was 6.4 years.

In 2 cases a history of intramuscular injection of blood could be elicited at intervals of eleven and seventeen years respectively prior to the delivery of the erythroblastotic infant in the first pregnancy. The indications for the administration of blood were prophylaxis against measles and poliomyelitis. Each of these 2 patients denied having previous pregnancies and/or abortions.

Finally, there is the group of twelve instances of erythroblastosis fetalis in the first born in which there is neither a history of transfusion or abortions. An additional 4 patients gave a history of early abortion. However, this can play no essential role in initiating transplacental isoimmunization which requires prolonged and slow administration of Rh positive fetal blood. It was in this group of cases that the suggestion was first made by Levine that these patients may have had intramuscular injections of blood many years previously. In the event that this antigenic stimulus can be excluded, these Rh- women can be considered as the group most susceptible to isoimmunization. Unfortunately there is as yet no known procedure which will differentiate this group from the group which is more resistant to isoimmunization.

Possibly fetal red blood cells escape into the maternal circulation in the course

\*This includes a second series of cases to be published by Levine and Rosenfield.<sup>34</sup>

of the second stage of labor when the placenta begins to separate \* Perhaps this serves to explain the increase of antibody content occasionally observed in the postpartum period However, parturition by itself can play no essential role in the group of cases discussed above and by the same token its importance in the mechanism of isoimmunization in general can be excluded

As indicated above there is no final proof for the concept that fetal blood cells find their way into the maternal circulation in every normal pregnancy However, this view seems to be compatible with serologic, clinical, and pathologic features of erythroblastosis fetalis Most workers hesitate to accept the theory of transplacental isoimmunization probably because of the traditional concept that the placenta presents a barrier which is absolutely impermeable to formed elements It would indeed be quite unique if nature repeatedly provided an absolutely perfect organ which in the course of its short life of 40 weeks attains a surface area of 70-120 square feet required for the nourishment of the fetus This would be all the more remarkable because of the rapid proliferation of the trophoblast which is endowed with invasive properties similar to that of the malignant cell

A discussion of the mechanism of transplacental isoimmunization cannot be complete without reference to the passage of maternal antibodies into the fetal circulation Recent attempts to associate prolonged intra-uterine action on the part of blocking antibodies with hemolysis and rapid action of agglutinins only at delivery with severe jaundice and toxicity have been shown to be premature <sup>36</sup> Although there are striking differences between agglutinins and blocking antibodies demonstrable in vitro, these differences lose their significance because in vivo both react with fetal blood suspended in a medium of plasma In any event, blocking antibodies in contrast to anti-Rh agglutinins are frequently demonstrable in the infant's circulation

The study of affected infants before and after a more or less complete replacement transfusion reveals the significant fact that appreciable quantities of blocking antibodies can still be demonstrated at the end of the replacement transfusion when the vast majority of the blood is not coated Presumably, large quantities of blocking antibodies are stored in the tissue spaces and hence, the continued blood destruction of any residual Rh+ fetal blood in the neonatal period Certainly, there seems to be little or no indication for the use of Rh+ blood in transfusing affected infants of Rh- mothers

With an antigenic stimulus acting over a period of several months in any one pregnancy and its renewal in the following pregnancies with Rh+ fetuses, it is not surprising that more than one variety of antibody is produced In a sense many Rh- women are subjected to hyperimmunization More recently, a third variety of antibody could be demonstrated in certain blocking sera which are characterized by a distinct prozone in the lower dilutions The antibody responsible for the prozone can be specifically absorbed after contact of the undiluted serum with Rh+ but not with Rh- blood (Levine and Wigod) †

\*Should the Rh factor be found in syncytial cells, their liberation in the postpartum period might also serve to immunize

†Cf Hill and Haberman <sup>37</sup>

These findings raise the question as to the nature of the globulin antibody produced by the immunized mother. One may speculate that the antibodies produced represent an ever changing configuration of the immune globulin without losing its characteristic specificity. It is not excluded that at times an antibody may be produced which has little or no capacity to pass the placental barrier. Possibly this may serve to explain the rare instances of entirely normal or mildly affected Rh+ infants delivered by intensively immunized Rh- women whose previous pregnancies resulted in severely affected infants (Chown<sup>35</sup>).

#### ADDENDUM

Claims were made recently that physiologic breaks in the villi could be demonstrated either by means of serial sections (Naeslund and Aren<sup>39</sup>) or by the presence of nucleated red cells of the erythroblastic fetus in intervillous spaces (Kline<sup>40</sup>). These observations require confirmation because of the inherent difficulties in providing histologic proof of physiologic breaks in the continuity of blood vessels of such delicate tissue as the placenta.

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# ERYTHROBLASTOSIS FETALIS IN NEGROID INFANTS

By A S WIENER, M D , AND I B WEXLER, M D

THE INCIDENCE of erythroblastosis among Caucasoids, as given by different authors, varies from 1 in 400 to 1 in 150 births <sup>1 2</sup> Despite the tremendous increase in interest in the disease since Levine et al <sup>3</sup> and Burnham<sup>4</sup> demonstrated the role of the Rh factor of Landsteiner and Wiener<sup>5 6</sup> in its pathogenesis, no reports have come to our attention concerning the appearance of the disease in Negroid infants We have recently encountered 3 such cases having unusual features, and these are the subject of this report

## CASE REPORTS

*Case 1* The patient, a male infant, was born at the St John's Hospital on October 8, 1946 This was the mother's first pregnancy and the entire period of gestation was uncomplicated There was no history of luetic infection and the Wassermann reaction of her blood was negative Delivery was spontaneous, at term, and the infant cried immediately The only abnormality noted at the delivery was a yellow discoloration of the vernix and amniotic fluid, while the cord was normal The birth weight was 7 pounds, 8 ounces

The infant appeared to be normal until the second day of life when moderate icterus of the skin and sclerae was noted There was no enlargement of the liver or spleen and the baby appeared to be otherwise well On the following day, however, jaundice became more intense and the infant vomited its feedings Laboratory findings at this time were as follows Hemoglobin concentration of the blood, 13 grams per 100 cc, red blood count, 4.5 million per cu mm, white blood count 9,500 per cu mm There were 2 normoblasts per 100 white blood cells on the smear The stool was yellow green and gave a negative test for blood Bleeding, coagulation, and prothombin times were normal The infant's red blood cell fragility was normal Both the mother and the baby were found to be Rh positive

The hemoglobin fell slowly for the next few days and on the sixth day of life was found to be 9.5 Gm. per 100 cc with a red count of 3.95 million per cu mm One eosinophile and 2 normoblasts were seen on the smear at this time Because of the falling hemoglobin and red count, a transfusion of 50 cc of group B, Rh-positive blood was given On the day following the transfusion, the hemoglobin had risen to 11.5 grams per 100 cc with the red count 3.92 million per cu mm This rise was not maintained, however, and a second transfusion of group B, Rh-positive blood was given Twelve days later the blood count had again fallen and now showed a hemoglobin of only 7.6 grams per 100 cc and a red count of 2.67 million per cu mm

Because of the obscure etiology of the patient's anemia and the poor response to transfusion therapy, the case was referred to us for further study Results of grouping and Rh-Hr tests on the father, mother, and infant were as follows

Blood of	Group	M N Type*	Rh Hr Type†
Father	AB	N	Rh.
Mother	O	MN	Rh <sub>0</sub>
Infant	B	MN	Rh <sub>0</sub>

From the Transfusion Division of the Department of Laboratories, and the Department of Pediatrics of the Jewish Hospital of Brooklyn

\* The types M N are not of clinical significance, but are included for the sake of completeness

† For heredity and nomenclature of the Rh-Hr types, see Wiener <sup>7 8</sup>

Titration of the alpha and beta antibodies in the mother's serum showed an anti-A titer of 15 units by the agglutination method and also 15 units by the conglutination method, the anti-B titer was 60 units by the agglutination method, by the conglutination method a titer of 600 units was obtained.\* These results show that the patient's anemia and poor response to transfusion were due to sensitization of the mother (group O) to the infant's erythrocytes (group B). On our recommendation, two small transfusions of group O red cells (plasma free) were then administered, after which the infant made an uneventful recovery. When seen again in the clinic at the age of 7 weeks, the infant weighed 9 pounds, 5 ounces and had no jaundice. Blood count was not done.

*Case 2* A Negress was referred to us with the following obstetrical history. Her first pregnancy in 1941 resulted in the birth of a normal male infant, who is living and well. The second pregnancy, in 1943, also gave rise to a normal male infant. Her third pregnancy terminated prematurely (8 months) with the birth of a male infant who became jaundiced at the age of 3 days. He was kept at the hospital for three weeks during which time the jaundice subsided. He was not transfused during his hospital stay and appeared well on arriving home. Shortly thereafter, however, he was seen to be pale. He was transfused immediately but lost weight, developed jaundice and died. The mother was now pregnant for the fourth time and the problem of the prognosis and treatment for the expected infant was presented to us. According to the mother, previous tests had shown her to be Rh negative and her husband Rh positive.

Results of grouping, Rh-Hr tests on the father, mother, and the two surviving sons were as follows:

Blood of	Group	M-N Type	Rh Hr Type
Father	B	M	Rh <sub>2</sub>
Mother	O	MN	Rh <sub>1</sub> Rh <sub>2</sub>
1st son	B	M	Rh <sub>1</sub> Rh <sub>2</sub>
2nd son	O	M	Rh <sub>1</sub> rh

These results show that the Rh-Hr types had nothing to do with the problem and that the previous report of the mother's Rh type was in error. Titration of the alpha and beta antibodies in the mother's serum showed an anti-A titer of 25 units by the agglutination method and a titer of 20 units by the conglutination method. The anti-B titers on the other hand, were 100 units by the agglutination method and 500 units by the conglutination method.

These findings support the diagnosis of erythroblastosis as the cause of death of the third infant, but with the B factor and not the Rh factor as the sensitizing agent. It must have been the first child who sensitized the mother, while the second escaped because it belonged to group O. The prognosis for the expected infant now depends upon its blood group. If it belongs to group O (50 per cent chance) it will not be erythroblastotic. If, however, it belongs to group B it will almost certainly have the disease. In such cases, the prophylactic injection of soluble A and B group substances into the infant by way of the umbilical vessels at the time of birth may serve to ameliorate the disease.<sup>10</sup>

*Case 3* The patient was a second child, female, born at term after a short labor. Pregnancy was uncomplicated and the Wassermann and Kline reactions of the mother's blood were negative. The baby weighed 9 pounds, 9 ounces at birth and was seen to be lethargic, pale, and appeared to have difficulty in breathing. A bradycardia of 110 beats per minute was present. There was no apparent jaundice, nor was the amniotic fluid or vernix discolored. A blood count done shortly after birth showed a hemoglobin concentration of only 7.7 grams per 100 cc, with a red cell count of 3.2 million per cu. mm., a white cell count of 86,000 per cu. mm., and 82 nucleated red blood cells per 100 white cells on the smear. The mother's blood was found to be A<sub>1</sub>MNRh<sub>1</sub>rh, and the baby's blood A<sub>1</sub>MRh<sub>1</sub>rh. There was therefore no known factor present in the infant's erythrocytes that was lacking from the mother's and no sensitization was possible.

\* By the method of titration used, the average normal titer with the agglutination technique is approximately 40 units. In nonsensitized individuals, the titer by the conglutination technique is lower, or at least not higher, than that by the agglutination technique.<sup>9, 11</sup>

to the A, B, or Rh-Hr factors. A 'coating' test (conglutination technic)<sup>11</sup> done on the baby's cells was negative, and tests done on the mother's serum for the presence of abnormal iso antibodies were negative.

Transfusion to combat the anemia was indicated and the type of blood to use presented a problem. In view of the possibility that the case might be one of isosensitization against a blood factor as yet undiscussed, it was decided to use the mother's washed red cells, because her erythrocytes could not contain the hypothetical immunizing factor. The child was therefore transfused with the washed red cells obtained from 100 cc. of the mother's blood resuspended in saline.<sup>12</sup> On the day following transfusion the hemoglobin had risen to 15 grams per 100 cc. Two days after birth jaundice was found to be present, and the hemoglobin had fallen to 11 grams per 100 cc. A blood smear at this time showed that there were 276 nucleated red blood cells per 100 white cells. Periorbital edema appeared and the transfusion of mother's blood, using the washed red cells obtained from 100 cc. of blood, was repeated. Response to this transfusion was good in that the hemoglobin now rose to 15.5 grams per 100 cc. and the red count to 5.67 million per cu. mm. However, there still were 216 nucleated red blood cells per 100 white cells on the smear. Within two days the nucleated red cell count had fallen to 12 per 100 white blood cells and five days following the second transfusion nucleated red cells were no longer present. Fragility tests done on the twenty-first hospital day were within normal limits. Numerous sickling preparations were negative, immediately and after twenty-four hours. Cultures of the nose and throat, stool, and blood failed to reveal any pathogenic organisms. X-ray of the skull showed no abnormalities. The child's clinical course was uneventful. She was discharged at the end of the fourth week weighing 5 pounds 9 ounces, with a hemoglobin of 10 grams per 100 cc. and a red cell count of 2.85 million per cu. mm. She had no jaundice at the time of discharge. When seen in the out-patient department, four weeks after discharge, she weighed 8 pounds, 1 ounce. Her hemoglobin had fallen to 9.1 grams per 100 cc. and her red count was 2.9 million per cu. mm., but otherwise she seemed well.

#### COMMENT

If the Rh factor played the same role in all races as it does in the Caucasian, one would expect the incidence of erythroblastosis in the various races to correspond with the frequency of the Rh-negative type. This expectation has apparently been fulfilled in the Mongolian race, since erythroblastosis is extremely rare amongst these peoples.<sup>13</sup> Among Negroids, with a frequency of Rh-negative individuals variously reported as between 5 and 10 per cent, one might similarly expect an incidence of erythroblastosis from one third to two thirds as high as in Caucasians. As we have already mentioned, however, erythroblastosis appears to be rare among Negroes, indicating that considerations other than the Rh type play an important role in the pathogenesis of the disease. It is particularly remarkable that in the 3 cases reported here with the clinical picture of erythroblastosis, none showed evidence of Rh sensitization. Somewhat similar observations have been made by Zuelzer.<sup>14</sup>

Recent observations indicate that the efficiency of sensitization in Rh-negative individuals depends in part upon the amount of Rh-positive blood inoculated into the body. For example, Rh-negative women, who at the first pregnancy have had stillbirths due to Rh sensitization, almost always show a history of having received a transfusion or intramuscular injection of blood some time in the past.<sup>15</sup> Furthermore, experiments done to produce Rh testing sera in male donors have shown that the great majority of Rh-negative individuals are readily sensitized by properly spaced injections of as little as 2 cc. of Rh-positive blood.<sup>16</sup> On the other hand, the observation that isosensitization by pregnancy appears to occur in only 1 out of 25 to 50 Rh-negative women, may be explained by the fact that during pregnancy or parturition only minute quantities of Rh-positive blood enter the maternal

circulation, and at intervals not necessarily optimal for the stimulation of antibody production. Another factor is the constitutional ability or lack of ability to be sensitized.<sup>17</sup> The situation is analogous to that existing in allergic diseases<sup>18</sup> or infections. An overwhelming dose of antigen or pathogenic organism will sensitize or infect all human beings, a minute dose will affect only the most susceptible. Accordingly, if one assumes that in Negroid races the placenta offers a better barrier to the passage of materials from the fetus to the mother, this would account for the rarity of erythroblastosis among these peoples. An alternative possibility is that among Negroids the frequency of individuals easy to sensitize is very low.

Past investigations have furthermore demonstrated that injections of soluble A and B substances in small amounts of secretions such as saliva may give rise to sensitization to these factors, while corresponding doses of red cells containing these substances are inadequate to sensitize.<sup>19</sup> Therefore, passage of soluble materials from fetus to mother may give rise to A and B sensitization under conditions which would be inadequate to cause Rh sensitization, because comparable small quantities of red cells would not be antigenic. It is therefore significant that 2 of our 3 cases can be explained on the basis of A and B sensitization. With regard to the third case, no evidence of isosensitization could be obtained, and if erythroblastosis is strictly defined as comprising those conditions in the newborn caused by isosensitization of the mother by an antigen in the fetal blood, then this case does not satisfy the conditions, according to our findings. However, there are a host of clinical conditions producing jaundice, anemia, hepato-splenomegaly and erythroblastemia in the newborn. Cases such as this, in which no satisfactory conclusion could be drawn demonstrate that there is still much to be learned in this most interesting field.

#### SUMMARY

Three cases of Negroid infants with clinical signs and symptoms resembling erythroblastosis fetalis were presented. In none of the cases was there any evidence of Rh sensitization. Two of the cases were apparently due to sensitization of the mother to the B agglutinin, in the third case no serologic incompatibility could be demonstrated. The possible significance of these observations was discussed.

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# AUTOHEMAGGLUTININS AND HEMOLYSINS WITH HEMOGLOBINURIA AND ACUTE HEMOLYTIC ANEMIA, IN AN ILLNESS RESEMBLING INFECTIOUS MONONUCLEOSIS\*

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ACUTE acquired hemolytic anemia is uncommon, but dramatic in its clinical picture and often disastrous in its outcome. In 1940, Dameshek and Schwartz<sup>1</sup> assembled about 100 cases reported in the literature since 1907, in which no definite etiology was evident. In addition, as cited by these authors, cases have been reported in which the anemia developed in association with definite or probable etiologic factors. These include malaria, infections with streptococci, staphylococci and certain anaerobic organisms, as well as tuberculosis, syphilis and ankylostomiasis, also pregnancy, lymphoma, leukemia, carcinomatosis, and, finally, various drugs, especially the sulfonamides but also arsenical preparations, phenylhydrazine and acetanilide. Hemolytic anemia has also been reported in association with atypical pneumonia of unknown etiology<sup>2, 3</sup> and its occurrence in patients who have received sulfonamides is well established.<sup>4</sup> Studies of these cases in regard to the presence of hemagglutinins and hemolysins have given varied results.

The case of acute hemolytic anemia with hemoglobinuria which is herewith reported is of interest because of the association of the unusual combination of auto-hemagglutinins and hemolysins occurring in the presence of morphologic changes in the white blood cells and heterophile agglutinins in the blood serum consistent with infectious mononucleosis, together with a positive Donath-Landsteiner reaction and the absence of evidence of syphilis.

The patient was observed during the acute stage of his illness by two of the authors (O J W and L B E) and more than two years later by the third author (R P S).

## REPORT OF CASE

*Present Illness* H J H, a 21 year old single white male was admitted to an Army hospital in the European Theater on January 9, 1945, with a three day history of bloody urine. Two weeks prior to admission he had developed an upper respiratory tract infection with a nonproductive hacking cough, headache, moderate nausea and anorexia. There was no vomiting until the day before admission when he had vomited once without hematemesis. On the evening of January 6, he first noted dark bloody urine, unaccompanied by urgency, frequency, dysuria or nocturia. The dark urine continued for the three days prior to admission. During this interval he developed a steady aching pain in the epigastrium and in the lumbar region. Generalized weakness and dyspnea on exertion became moderately distressing, but he continued on duty until admission. At no time did he experience chills, fever, icterus, or symptoms referable to the lower intestinal tract. His weight had decreased from his usual 128 pounds to 105 pounds on admission.

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From the Thorndike Memorial Laboratory and the Second and Fourth Medical Services (Harvard) of the Boston City Hospital, and the Department of Medicine, Harvard Medical School.

\* This patient was observed from January to March, 1945, in an Army Hospital in the European Theater of Operations and subsequently in 1947 in a Veterans Administration Hospital.

*Past History* In 1937 he had an uncomplicated appendectomy, in 1942, a hemorrhoidectomy. On July 21, 1944 he was admitted to an Army General Hospital with lymphadenitis of the right arm and was discharged well on August 10. On August 24, 1944 he developed impetigo and was treated with ammoniated mercury and ultra violet light until September 16, 1944 when he was discharged as well. From September until the present illness, approximately four months later, he enjoyed good health. In November, 1944 his attention had first been called to asymptomatic enlargement of his finger tips which had not changed since, to his knowledge.

During the few days immediately preceding entry, he had developed a generalized pruritic skin lesion which, on admission, proved to be scabies. During his three years of army service he had never had tropical service, and he had not been exposed to any known hemolytic agents. He had received no sulfonamide drugs for at least six months prior to his illness. His diet had been adequate. Alcohol had not been used habitually nor in excess immediately prior to the present illness. There was no history of recent disease. He had never experienced an illness similar to the present malady, nor had he ever been seriously ill.

*Family History* No similar illness was known. The familial history was noncontributory.

*Physical Examination* Temperature 99.4 F, pulse 110, respiration 20, blood pressure 120 mm of mercury systolic and 70 mm diastolic. He was ambulatory and complained chiefly of weakness. He did not appear acutely ill but was pale and slightly icteric. Scabetic furrows and scratches were present over the trunk and extremities. (Subsequent treatment for scabies led to rapid clearing.) The mucous membranes were moderately pale but presented no evidence of hemorrhage or ulceration. There was a nontender left infra-auricular lymph node approximately 1 cm in diameter. No other lymphadenopathy was detected. The thyroid was not enlarged. The chest was symmetrical and the examination of the lungs was normal. The heart was not enlarged. There was a strong apical impulse and a blowing apical systolic murmur was heard on auscultation. The abdomen was scaphoid with an indefinite epigastric tenderness. The liver and spleen were palpable only on inspiration and were tender. The kidneys could be palpated but were not enlarged or tender. The genitalia were normal. The fingers presented a striking terminal enlargement characteristic of clubbing, the nails appeared otherwise normal. The toes did not show similar changes. The extremities were not cold or sweaty. Examination of the long bones, skull, the muscular and the neurologic systems was not remarkable. There was no demonstrable edema or evidence of dehydration.

#### LABORATORY DATA

*Methods Employed* References are given below to the technical methods employed in the laboratory examinations.

*Urine Examination* hemosiderin (Rouss technique<sup>5</sup>), porphyrins<sup>5</sup>, alkapton bodies<sup>5</sup>, bile (Rosenbach's modification of Gmelin's test<sup>6</sup>), urobilinogen (Wallace and Diamond modification of Ehrlich aldehyde test<sup>6</sup>), hemoglobin in plasma and urine<sup>7</sup>, indican (Obermayer's test<sup>6</sup>).

*Blood Examination* hemoglobin (alkaline hematin method using a Coleman spectrophotometer), red cell fragility<sup>8</sup>, erythrocyte count (Hayem's solution), platelet count<sup>8</sup>, bleeding time (Duke's method<sup>8</sup>), clotting time<sup>8</sup>, hematocrit and sedimentation rate (Wintrobe method<sup>8</sup>), test for sickle cell trait<sup>8</sup>.

*Serologic Examination* heterophile agglutination, presumptive test (Davidsohn technique<sup>10, 11</sup>), Donath-Landsteiner<sup>9</sup>, hemolysis test with acidified serum<sup>7</sup>, cold hemagglutinins<sup>12</sup>.

Other methods employed were either too well known to require comment or are described in the text.

Hematologic data during this hospitalization are given in table 1 and figure 1.

#### RESULTS

1. *Anemia* The anemia was obviously hemolytic in type as evidenced by extreme hemoglobinuria, and became profound on the day following admission, the hemoglobin dropping from 12.2 grams per cent at 10:00 A. M. to 5.9 grams per cent by 3:00 P. M. of the same day. At this time blood platelets were 232,000, bleeding time one minute, clotting time five and one-half minutes (capillary tube), and clot re

Date	HGB	RBC	H BC X 1000	Differential					Hema to crit	Serum Bili rubin	Hetero phile Aggl	Cold Aggl (Group "O" Cells)	Urine				Remarks
	Gm %	mil		Stab	Ser	Eos	Bas	Lym	Mono	%	%	%	Hgb	Alb	Bile	Urob- lino- gen	
1945																	
Jan																	
9	12.2	3.30	24.3	32	2			63	2				4+	3+	1+		NPN 28 mg %, Bl Sugar 112 mg %, lymphocytes atypical
10 (AM)													0	2+	1+		Acid hemolysis and sickling neg,
10 (PM)	5.9	clumped	15.7							22		1 256*	0	1+	1+		Donath-Landsteiner positive
11	7.1	2.24	21.0	38				52		25		1 128*	0	1+	1+		Blood Kahn and Wassermann neg
12	8.6	2.90	8.3	48				49		26			4+	3+	1+		Fragility of RBC normal
13	10.6		21.3										0	1+		1 40	
14	9.4	2.74	15.6	36	2			56	2	28		1 64	0	1+	0		Sed of RBC 1 mm /min
16	9.8	2.83	13.6	34	3			51	9	29			0	0	0	1 40	
18	9.0	2.81	9.5	38	4			48	5	28		1 64	0	0	0		
19	7.7	2.21	15.6	44				50	3				0	0	0	1 160	Total prot 9.1 Gm %, alb 5.8, glob 3.3
21	5.6	1.63	9.7	34				64					0	0	0	1 160	
24	9.1	2.69	10.8	55	4			34	3				0	0	0		
Feb																	
1	9.4	3.16	8.5	65	2			28	3				0	0	0	1 20	
3	10.0	2.83	6.6	58	3			36	2			1 64	0	0	0	0	
10	11.2	3.40	7.0	71	1			24	2	30		1 256	0	0	0		Fragility of RBC normal
13	11.0	3.52	8.5	65	1			29	1	35		1 128	0	0	0		BMR +22
17	13.0	3.9	5.2	50	1			44	1	38		1 128	0	0	0		Donath-Landsteiner pos, MCV 92, MCH 31, MCHC, 28
Mar																	
7	11.4	3.75	9.2	69	1			26	3				0	0	0		See Tests—Tables 4 and 5
12	13.3	4.05						2.2		37			0	0	0		
1947																	
Feb	16.0	4.50	11.0	63	1			26	10			neg	0	0	0		Donath-Landsteiner neg

\* 1.64 Patient's cells

traction normal The osmotic fragility test of the patient's red blood cells was identical with the control on the second hospital day, on three subsequent occasions within the next month, and two years later, in 1947 During the first two days difficulty was encountered in performing erythrocyte counts due to clumping of the cells in the pipet at the room temperature of approximately 20 degrees Cent

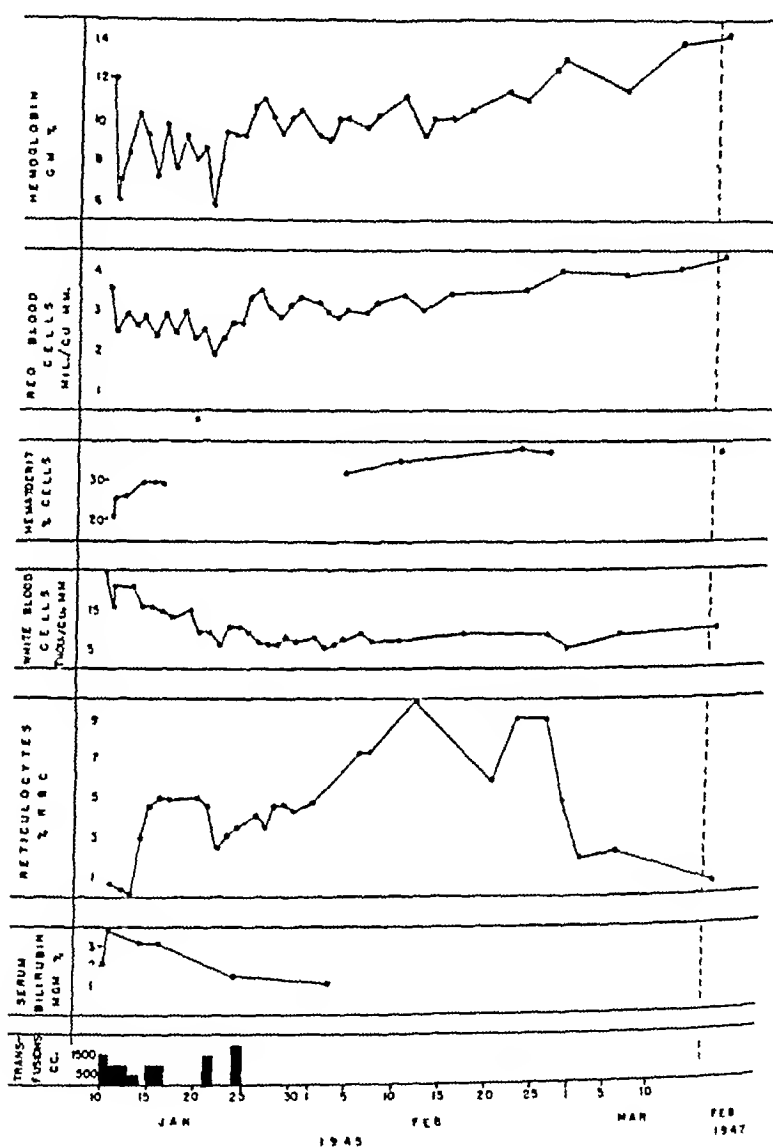


FIG 1

grade Subsequently warm saline solution was employed as a diluent and counts were then satisfactorily made

The course of the anemia is shown in figure 1 Therapy consisted of transfusions of whole blood and concentrated red cells as described below, iron therapy and a high caloric, high vitamin diet

2. *Morphology of the Blood* Examination of the morphology of the erythrocytes was not striking The anemia was essentially normocytic and normochromic and the red blood cells appeared morphologically normal No nucleated red cells were

observed. The reticulocytes rose during the first and second week as shown in figure 1. There was an initial leukocytosis on January 10 of 24,300, with a differential of segmented polynuclear cells 32 per cent, eosinophiles 2 per cent, lymphocytes 64 per cent, monocytes 2 per cent. The lymphocytes were typical of the cells seen in infectious mononucleosis; the majority conformed to Downey's Class I<sup>13</sup>, a small percentage were Class II and III. The leukocyte count did not exceed 10,000 per cubic millimeter after the second week and the relative lymphocytosis with the atypical lymphocytes gradually disappeared.

3 *Hemagglutinins and Hemolysins* On admission hemagglutinins in the patient's serum with red cells of blood group O were demonstrated in a dilution of 1:256 at 4-6 degrees Centigrade. Clumping also occurred at 10 and 20 C and persisted when the test was carried out in the incubator at 37 C. When preparations which previously had been chilled at 4-6 C were subjected to a temperature of 37.5 C for one hour the clumping persisted and hemolysis occurred. There was no hemolysis at 4-6 C. After subjecting the patient's serum to a temperature of 56 C for inactivation of serum complement hemolysis was not observed but agglutination reactions were

TABLE 2.—*Presumptive Donath-Landsteiner Test\**

Temperature Conditions	Plasma Hemoglobin of Defibrinated Blood	
	Patient	Control
	mg per 100 cc	mg per 100 cc
Room temperature	70	2.1
1 hr at 37.5 C	90	2.1
20 min at 4-6 C, then 1 hr at 37.5 C	370	2.1

\* Two cc samples of defibrinated blood from the patient and a normal control were treated as indicated.

maintained. The hemagglutination against red cells of blood group O dropped to 1:64 by January 14, where it remained throughout this hospital admission.

The presumptive (table 2) and more complete Donath-Landsteiner tests (table 3) were strongly positive on admission; guinea pig serum was not available for addition as a source of complement to the heat inactivated serum. Donath-Landsteiner tests were repeated seven weeks later, with the same positive result. The Kahn and Wassermann tests were negative on four occasions during this month, and again in December, 1945 and January, 1947, and there was no history or physical sign of syphilis.

The relationship between chilling of the patient and the development of increased hemoglobinemia and hemoglobinuria was explored but unfortunately without conclusive results. During his hospitalization the patient was exposed to the usual environment of a drafty Nissen hut ward heated by coke stoves in England in the winter. During the first day of his stay he was a 'bed patient,' but was rather recalcitrant and difficult to keep in bed. The opportunity for chilling undoubtedly existed. No hemoglobinuria was present after the fourth hospital day. Obvious hemoglobinemia was observed on admission and on several occasions during the

first four days of hospitalization. Subsequent observations of the plasma showed moderate hemoglobinemia, above the level of controls, until January 16. Quantitative results are not reported because of difficulty in preparing a proper benzidine reagent.<sup>7</sup> An attempt was made to test the effect of chilling by placing the patient's arm in ice water on March 13. The experiment had to be terminated in fifteen min

TABLE 3 — *Donath-Landsteiner Test*

Tube number	RBC—5% suspension in saline 0.5 cc	Serum—fresh 0.5 cc	Serum†—heated 5 min at 56° 0.5 cc	Degree of hemolysis	Treatment of mixtures
1	P*	P		0	Incubated 1 hr at 37.5 C
2	C*	P		0	
3	P	C		0	
4	C	C		0	
5	P	P		2+	Chilled 20 min at 4-6 C, incubated 1 hr at 37.5 C
6	C	P		2+	
7	P	C		0	
8	C	C		0	
9	P		P	0	Chilled 20 min at 4-6 C, incubated 1 hr at 37.5 C
10	C		P	0	
11	P		C	0	
12	C		C	0	

\* P = Patient, C = Normal Control, red cells washed three times in physiologic saline at room temperature. Both blood samples Group A.

† Guinea pig serum was not available as a source of complement.

TABLE 4 — *Observations Following Immersion of Right Arm in Ice Water (March 12, 1945)*

	Before test	Right arm immersed 10 min		Right arm blood after chilling 15 min
		Right arm blood	Left arm blood	
Hemoglobin, Gm %	13.28	12.96	12.64	12.16
RBC, millions	4.05	4.02	3.88	3.74
Hematocrit, %	37	37	37	35
Plasma Hgb, mg per 100 cc	48	58	59	70
Reticulocytes, %	2.2	2.2	2.4	2.0
Urine hemoglobin	Neg			Neg
Urobilinogen	1.40			1.40

utes because the patient fainted. No hemoglobinuria developed and the changes in the red blood cell count and level of free plasma hemoglobin were equivocal (table 4). The high levels for plasma hemoglobin in all control samples were considered to result from the benzidine reagent as mentioned above, precautions were taken to prevent hemolysis in obtaining the blood samples.

As shown in table 5, the hemolysin was absorbed from the patient's serum at 4-6 C by normal group A red blood corpuscles and was not removed by three washings

in saline at room temperature. The hemolysin was active only in the presence of complement since inactivation of normal serum at 56 C prevented the hemolysis which occurred with fresh unheated serum.

4 *Heterophile Antibody* On admission, the heterophile agglutination was positive in a dilution of 1:1024, and remained at that level 10 days after admission on January 19. On February 10 it had fallen to 1:128, at which level it persisted throughout his stay in this hospital. Unfortunately, it was not possible to absorb the serum with guinea pig kidney or ox cells to determine the variety of heterophile antibody.<sup>11</sup>

TABLE 5 — *Absorption of hemolysin (March 12, 1945)*

Tube number	RBC—5% suspension in saline 0.5 cc	Serum—fresh 0.5 cc	Serum—heated 5 min 56 C 0.5 cc	Treatment of mixtures	Hemolysis
1	C*	P*		Chilled 20 min at 4-6 C, incubated 1 hr at 37.5 C	+
2	C	P		Chilled to 4-6 C, centrifuged, serum and cells separated at 4-6 C = P <sub>2</sub> and C <sub>2</sub>	o
3	P fresh	P <sub>2</sub>		Chilled 20 min at 4-6 C, incubated 1 hr at 37.5 C	o
4	C <sub>2</sub> (Washed 3 times in saline, 25 C)	C		Incubated 1 hr at 37.5 C	+
5	C fresh	P <sub>2</sub>		Chilled 20 min at 4-6 C, incubated 1 hr at 37.5 C	o
6	C <sub>2</sub> (Washed 3 times in saline)		C	Incubated 1 hr at 37.5 C	o

\*P = Patient, C = Normal Control, red cells washed three times in physiological saline at room temperature. Both blood samples were Group A.

5 *Blood Grouping and Transfusion Therapy* Upon admission blood grouping with rabbit immune sera and washed red cells of the patient at 37 C indicated that the patient belonged to blood group 'O'. The same group was recorded on his Army Identification tag. Later, it was demonstrated both by us and the North East London Blood Supply Depot that the patient was, in reality, blood group A, as demonstrated by high titer grouping serums. No studies were made to determine the possible subgroups A<sub>1</sub> and A<sub>2</sub>.

In spite of hemagglutination demonstrated at room temperature and at 4-6 C, it was evident that transfusions were necessary to combat the rapid fall of the hemoglobin. Since the initial blood group was considered to be group O, 1500 cc of group O blood, warmed to body temperature, was given on the evening of



# UNUSUAL PICTURE IN POSSIBLE INFECTIOUS MONONUCLEOSIS

January 10 There was no reaction The Rh blood group could not be determined at this time since anti-Rh serum was not available

Transfusions were continued and a total of 7500 cubic centimeters of blood was given between January 10 and 21 \* The patient occasionally complained of pain in the right and left upper quadrants following transfusion but no evidence of increased hemolysis could be demonstrated as having occurred Only group O blood without Rh determination had been given up until January 24 At that time anti Rh serum became available and a mixture of Rh negative and positive cells was demonstrated in the patient's blood Rather than speculate which were the undetermined Rh blood which had been given, concentrated Rh negative group A cells from 2000 cc of whole blood were suspended in physiologic salt solution and administered at room temperature on January 24 A transient slight pyrogenic reaction followed No further transfusions were given prior to evacuation to the United States

6 *Urinary Findings* The admission urine specimen, January 9, was port wine in color, showed 3 plus albumin, a 4 plus reaction for hemoglobin and a 1 plus bile test Tests for sugar, acetone, porphyrin, indican, alkapton and hemosiderin were negative Urobilinogen and urobilin were not determined on this specimen Numerous granular casts were present in the centrifuged specimens but no white blood cells, red blood cells or blood cell casts On the second hospital day the urine was dark but not red It contained a 2 plus albumin, 2 plus bile, but the test for hemoglobin was negative and the sediment was negative Spectroscopic examination of the specimen by the North East London Blood Supply Depot, Luton, England, showed an increase in urobilinogen, urobilin and bile but no evidence of porphyrins On the fourth hospital day the morning specimen showed a 4 plus reaction to benzidine, an afternoon specimen was free of hemoglobin and no specimen thereafter contained either hemoglobin or bile Urine urobilinogen was positive in 1:40 dilution on the fifth and eighth hospital days and remained positive in 1:80 to 1:160 dilution until January 24 when it became entirely normal Concentration tests and fractional phenosulphonphthalein excretion tests were normal

7 *Miscellaneous Tests* In addition to the laboratory examinations already discussed, numerous other tests were carried out and are shown in table 1 All, including chest roentgenograms on admission, were normal or unrevealing as to the nature of the hemolytic process

*Course in Hospital* During the first two weeks the patient was critically ill, but throughout this period, as well as later, was active and loathe to stay in bed By January 22, the anemia had ceased to progress and from then on steady improvement occurred A peak reticulocytosis of 9.8 per cent was found on February 10 During the period of marked anemia the patient exhibited a low-grade fever, reaching 101 F on two occasions As the red blood cell level was restored, the temperature returned to normal and after February 8 never exceeded 99 Fluid intake and output were satisfactory The patient gained 5 pounds during the two month hospitalization

\* All blood was generously supplied by the N E London Blood Supply Depot, Luton, England

*Subsequent Course and Present Condition* The patient was evacuated to a hospital in the United States as an ambulatory patient on March 12, 1945. There, in April, the heterophile test was still positive (titer unavailable) and serum phosphorus, calcium and alkaline phosphatase determinations were normal, as was an electrocardiogram. Roentgenograms showed widespread slight osteoporosis of the tibiae, fibulae, lumbar vertebrae and skull. Biopsy of a lymph node from the right inguinal region was reported as showing subacute inflammation, not inconsistent with infectious mononucleosis. He was transferred to another hospital in December, 1945 when he developed a fissure of the rectum and hemorrhoids and was operated upon uneventfully. At this time, the sedimentation rate was 32 millimeters per hour, whole blood chlorides 479 milligrams per cent, CO<sub>2</sub> combining power 61 volumes per cent, serum phosphorus 3.8 milligrams per cent, serum calcium 11.3 milligrams per cent, and urea clearance test was 72 per cent of normal. Sternal puncture was negative and a modified Donath-Landsteiner test was now negative. During this entire period he remained weak, underweight, developed pains in his legs of increasing severity, clubbing of the toes was observed at that time with an apparent increase in the clubbing of the fingers. He was discharged from the Army in January, 1946.

On January 21, 1947, he entered a Veterans Administration Hospital for further study, where he remained until March 7. His complaints were persistent aching in the legs, a pressure sense in the rectum with frequent mucoid defecations, listlessness, nervousness and chronic fatigue. On physical examination he appeared somewhat agitated, there was audible hyperperistalsis and tenderness upon palpation of the rectum and prostate, clubbing of the fingers and toes, cold, clammy hands and feet, and 'marked' adenopathy in the inguinal, femoral and posterior cervical regions.

Extensive laboratory studies were made at this time.\* They are shown in table 1. In essence, all hematologic studies were then within normal limits. Six serologic tests for syphilis were carried out and were negative (Kahn, Kolmer, Kline, Eagle, Hinton, Mazzini). Stool examination showed mucus but no blood, parasites or ova. Sigmoidoscopy was normal.

Roentgenograms of the chest including bronchograms were normal. The hands and feet and long bones showed clubbing of the terminal phalanges of all fingers and toes, and there was expansion of the carpal and metacarpal bones with coarseness and trabeculations and thickening of the cortex. The skull had a somewhat granular appearance. A barium enema revealed a spastic colon.

### DISCUSSION

Did this patient have infectious mononucleosis? The heterophile antibody decreased coincident with the decrease in serum hemagglutinins but at all times it was positive in much higher dilution than was the hemagglutinin. Belk,<sup>14</sup> in studying a patient convalescent from infectious mononucleosis, found not only heterophile agglutinins and hemolysins against sheep, horse, rabbit and pig cells, but also autoagglutinins which were active below 10 Centigrade, but not at 37. He suggested that a nonspecific stimulus in this disease might result in a widespread production of antibodies. In a study of cold agglutinins, Favour<sup>15</sup> found them present in four of ten cases of infectious mononucleosis, with a maximum titer of 1:180. Springyarn et al.<sup>16</sup> have demonstrated them in seven cases of this disease. In their search for cold hemagglutinins in various disease states, Finland and his associates<sup>12</sup> found none in the three cases of infectious mononucleosis which they investigated. Davidsohn<sup>17</sup> found the titer of isoagglutinins normal in 44 cases of infectious mononucleosis.

The morphologic white blood cell picture of hemolytic anemia is usually described as an absolute and relative polymorphonuclear leukocytosis. An absolute and relative lymphocytosis, as seen in our patient, is unusual, and the presence of atypical lymphocytes, characteristic of those found in infectious mononucleosis is

\* Many of the tests were generously made by the Blood Laboratory of the Pratt Diagnostic Hospital, Boston.

even more unusual Dameshek<sup>4</sup> has reported an instance of hemolytic anemia in a patient with infectious mononucleosis who had also received sulfadiazine. A potent iso- and autohemagglutinin especially active at ice box and room temperatures was present in the serum. He felt that the drug played an important role in the production of the anemia. With this exception, no instances of hemolytic anemia occurring in infectious mononucleosis have come to our attention. In their review of hemolytic anemias Dameshek and Schwartz<sup>1</sup> cited none. In his monograph on infectious mononucleosis Bernstein<sup>18</sup> stated that "anemia of any appreciable degree does not appear unless associated with some complicating feature such as hemorrhage of dietary deficiency."

The evidence in this case suggests the possible diagnosis of infectious mononucleosis although it cannot be proved. It was not possible to classify the heterophile antibody by absorption studies.<sup>11</sup> Although there was a history of an upper respiratory infection two weeks before the first hospital admission, there was insufficient evidence to establish a diagnosis of atypical virus pneumonia. Since no other etiologic factor for his hemolytic anemia was evident, a causal connection between his anemia and the development of abnormal serum antibodies is suggested, possibly related to a disease resembling infectious mononucleosis.

Another feature of interest is the positive Donath-Landsteiner reaction. Such reactions usually have been found in cases of paroxysmal hemoglobinuria associated with syphilis,<sup>9</sup> although a few instances of its occurrence in hemolytic syndromes in the absence of syphilis have been reported. In our patient there was no evidence of syphilis. Stats and Wasserman,<sup>19</sup> who estimated that 92 per cent of cases showing a positive Donath-Landsteiner reaction have an associated syphilis, were of the opinion that fundamentally different antibodies are responsible for cold hemagglutinins and a positive Donath-Landsteiner reaction. They found but one case in the literature in which both have been reported<sup>20</sup> and refer to one further case with a positive Donath-Landsteiner reaction in which cold hemagglutination at from 0 to 3 Centigrade was observed.<sup>21</sup>

A relationship between the clubbing of the digits and the blood changes is unlikely. The presence of cold hemagglutinins and the occurrence of hemolytic manifestations have been described in patients with peripheral vascular disease, especially of the vasospastic type such as Raynaud's disease.<sup>19</sup> Peripheral osteoarthritis is generally considered to be related to abnormal circulation to the bone, but the association of clubbing with peripheral vascular disease is rare. In the present instance the clubbing preceded the acute hemolytic crisis and progressed after the hematologic abnormalities had disappeared and the hemagglutinins had diminished to a very low titer. This patient showed no evidence of peripheral vascular disease other than a tendency toward moderately cold and cyanotic hands and feet, with hyperhidrosis of the palms developing after his acute hemolytic episode. It is hardly tenable to relate the association of the bone changes to the hemolytic anemia or an illness resembling infectious mononucleosis unless the hypothesis is advanced that there was an underlying circulatory dystrophy with a chronic but minimal production of hemagglutinins which gave rise to a hemolytic crisis when the concentration of these antibodies was increased by the acute disease process.

## SUMMARY AND CONCLUSIONS

A case is reported of a young man with acute hemolytic anemia and hemoglobinuria who presented an initial blood picture consistent with infectious mononucleosis, associated with a heterophile agglutination test positive in high dilution, auto-hemagglutinins, active in the cold, at room temperature and at 37 Centigrade, a hemolysin active at 37 C after chilling, requiring the presence of a thermolabile component of serum for hemolysis, a positive Donath-Landsteiner test but no evidence of syphilis. In addition there was clubbing of the digits with certain other roentgenologic changes in the bones, absence of any other etiologic factors known to be concerned with such anemia, uneventful improvement under massive transfusion therapy, with apparent recovery from his hematologic disorder when studied two years later.

## ACKNOWLEDGMENT

Our thanks are due to Dr Maxwell Finland and Dr Philip F Wagley for suggestions in preparing this report. We are especially indebted to Dr T Hale Ham for his patient and critical advice and encouragement.

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## PRIMARY NONFAMILIAL HEMOLYTIC ANEMIA

By J M STICKNEY, M D , AND FRANK J HECK, M D

**A**LTHOUGH patients with hemolytic anemia are not numerous, they continue to be a problem of special interest and great difficulty. In the majority of cases the disease is of the familial or congenital type. The commonly accepted criteria for the diagnosis of congenital hemolytic anemia include the presence of a microspherocytic blood picture with an increase in signs of regenerative activity, increased fragility of the erythrocytes in varying concentrations of hypotonic saline solution, splenomegaly, an elevated value for indirect serum bilirubin with an increased excretion of fecal urobilinogen, and a history of anemia, icterus, splenomegaly or increased fragility of erythrocytes in other members of the patient's family.

In the differential diagnosis of the different types of hemolytic anemia, the question not infrequently arises as to whether an individual instance of the disease should be regarded as belonging to the congenital or familial type or to the acquired type. As Watson pointed out, there has been a tendency to regard all instances of "primary hemolytic jaundice as of familial or congenital type." There are, however, no clear-cut criteria to which all writers on the subject agree. In some cases in which the family history is negative but other criteria are present, the disease is classified as acquired. It must be admitted that a negative family history is not a definite indication that the disease is of the acquired type since actual investigation of close relatives may reveal such changes as increased fragility of the erythrocytes in the absence of other findings.

In the years 1942 through 1946, at the Mayo Clinic, splenectomy was performed in 22 cases of hemolytic anemia in which no positive family history could be obtained. These 22 cases are the object of our special interest.\* As far as could be determined, the hemolytic syndrome in these cases was not secondary to any toxic, infectious or poisonous agent and was not symptomatic and part of a primary disease such as lymphoblastoma, leukemia or hepatic cirrhosis.

We have divided these cases into two groups which happen to be equal in number. In the first group, either microspherocytosis or increased fragility of the erythrocytes or both were found. In the second group, such evidence was not present. The groups are summarized in tables 1 and 2.

### REPORT OF SELECTED CASES

*Case 3* The patient was a married woman, aged 35. There was no family history of anemia, jaundice or splenomegaly. In November, 1940 she complained of weakness and malaise. She was yellowish and was found to be anemic. On March 10, 1941, the erythrocytes numbered 790,000 and the leukocytes numbered 77,750 per cubic millimeter of blood. Many transfusions were given over a period of one month with moderate benefit. Physical examination disclosed that the spleen was enlarged and extended about 1½ inches below the costal margin.

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\* In the years 1942 through 1946, a diagnosis of congenital hemolytic icterus was made in 115 cases at the Mayo Clinic. Splenectomy was performed in approximately 90 of these cases.

When she was admitted to the hospital on May 1, 1941, the hemoglobin value was 11.05 Gm per 100 cc, the erythrocyte count was 3,140,000, the leukocyte count was 7,500 and the reticulocytes numbered 17.6 per cent. Examination of a blood smear showed the picture of congenital hemolytic icterus (microspherocytosis). The serum bilirubin value (indirect reaction) was 0.9 mg per 100 cc of serum. The brom-sulfalein test for liver function did not show any retention of the dye. The fragility of the erythrocytes was normal, initial hemolysis occurred at a concentration of sodium chloride of 0.44 per cent with complete hemolysis at 0.32 per cent. Splenectomy was performed May 5, 1941, the spleen weighed 310 Gm. The patient had a stormy convalescence but was dismissed June 21, 1941, without having been given any transfusion. At that time, the hemoglobin value was 10.6 Gm and the erythrocyte count was 3,700,000.

TABLE 1 — Hemolytic Anemia with Significant Microspherocytosis

Case	Age	Sex	Before splenectomy					After splenectomy			
			Hemo- globin, Gm per 100 cc	Erythrocytes		Retic- ulo- cytes	Gall stones	Time fol- lowed	Hemoglobin, per 100 cc	Erythro- cytes, No per cu mm	Result
				No per cu mm	Fragility* %						
	yr				%	%		mos			
1	26	F	3.9	1,390,000	0.46-0.38	25	0	13		4,100,000	Improvement
2	30	F	6.0	1,530,000	0.50-0.38	62	0	30		4,000,000	Excellent
3	35	F	11.0	3,140,000	0.44-0.32	18	+	12	8.3 Gm	2,740,000	Poor†
4	39	F	8.4	2,520,000	0.50-0.38	51	+	1	10.8 Gm	4,520,000	Improvement
5	39	F	8.3	2,710,000	0.46-0.32	32	0	9	84%	3,537,000	Excellent
6	54	F	8.4	2,900,000	0.46-0.36	24	+	26	88%		Excellent
7	59	F	6.3	3,850,000	0.48-0.36	3	0	12	12.5 Gm	3,850,000	Excellent
8	60	M	6.8	2,000,000	0.50-0.34	37	0	24	90%	5,000,000	Excellent
9	61	F	4.9	1,640,000	0.66-0.40	40	0	4	13.6 Gm	3,760,000	Excellent†
10	65	F	6.0	1,520,000	0.50-0.36		0				Died 8th post operative day Trans- fusion reac- tion
11	74	M	4.3	1,150,000	0.50-0.36	27	0	24	60%	2,800,000	Fair

\* In hypotonic solution of sodium chloride

† Reported in detail in text

When the patient returned to the clinic on August 11, 1941, the hemoglobin value was 10.6 Gm, the erythrocyte count was 3,430,000 and the reticulocytes numbered 6.5 per cent. On September 19, 1941, the hemoglobin value was 10 Gm, the erythrocyte count was 2,610,000 and the reticulocytes numbered 24.1 per cent. The fragility of the erythrocytes had increased, initial hemolysis occurred at 0.5 per cent and was complete at 0.36 per cent. On May 9, 1942, the hemoglobin value was 8.3 Gm, the erythrocyte count was 2,740,000 and the reticulocytes numbered 28.8 per cent. The value for the indirect serum bilirubin was 1.8 mg. Examination of blood smears revealed typical microspherocytosis and increased regeneration of erythrocytes.

In a letter dated February 7, 1945, the patient stated that her hematologic picture was about the same as it had been before splenectomy was performed.

Case 3 illustrates the failure of splenectomy to relieve the anemia. Of interest in this case are the presence of normal fragility prior to splenectomy and an increase in fragility after removal of the spleen. Despite the absence of a family history of hemolytic anemia in this case, the blood picture was considered "typically that of hemolytic icterus" by several observers.

*Case 9* A married woman, aged 61, came to the clinic on December 3, 1946. She had been perfectly well until the previous summer, when she had noted a loss of strength and loss of appetite. In the fall of 1946, she had become very thirsty. On October 31, she had been admitted to a hospital because of diabetic coma. The blood sugar value was 310 mg per 100 cc. The presence of diabetes had not been recognized previously. Hematologic examination had disclosed severe anemia, the hemoglobin value had been found to be 5.6 Gm. She had not been jaundiced previously and there was no family history of anemia, jaundice or splenomegaly. The diabetes had been controlled by dietary measures and by the administration of insulin. She had received eight transfusions of blood, 500 cc at each transfusion. The last transfusion had

TABLE 2—*Hemolytic Anemia without Significant Microspherocytosis*

Case	Age	Sex	Before splenectomy						After splenectomy			
			Hemo- globin, Gm per 100 cc	Erythrocytes		Retic- ulo- cytes	Gall- stones	Time fol- lowed	Hemoglobin, per 100 cc	Erythro- cytes, no., per cu mm	Result	
				No. per cu mm	Fragility*							
	Yrs				%	%		mos				
12	4	F	8.1	2,770,000	40-0	28	13	0	24	5.8 Gm	2,380,000	Poor Ex- plored for accessory spleen
13	13	M	10.3	3,270,000	44-0	30	10	+	24	10.6 Gm	2,980,000	Poor†
14	19	F	4.3	800,000	46-0	36	40	0	12		3,750,000	Excellent
15	21	F	8.8	2,520,000	44-0	34	32	0	23	78%	4,120,000	Excellent†
16	24	F	11.9	4,100,000	44-0	32	5	+	4	13.8 Gm		Improvement
17	33	M	6.3	2,220,000	44-0	32	17		40	14.8 Gm		Excellent†
18	34	F	11.6	3,350,000	42-0	30	12	+	18	78%	3,400,000	Fair One episode of jaundice
19	40	F	4.9	1,560,000	44-0	30	30	0	4	11.0 Gm		Died of intes- tinal ob- struction
20	46	F	8.1	3,850,000	48-0	28	3	+	1	11.05 Gm	4,920,000	Unknown
21	54	F	5.5	1,340,000	44-0	30	30	+	12	12.9 Gm	4,050,000	Excellent
22	59	F	4.7	1,520,000	44-0	32	31	0	4	10.2 Gm	4,000,000	Died of serum hepatitis

\* In hypotonic solution of sodium chloride

† Reported in detail in text

‡ Course following splenectomy reported through courtesy of Dr. C. J. Watson

been administered on November 29. Despite the lack of clinical evidence of transfusion reactions, the anemia had not improved.

When the patient came to the clinic on December 3, the hemoglobin value was 4.9 Gm, the erythrocyte count was 1,640,000 and the leukocyte count was 11,200. Examination disclosed moderate icterus. The spleen was greatly enlarged and extended downward to the crest of the ilium. Examination of blood smears disclosed marked microspherocytosis, very active regeneration of erythrocytes, and 40 per cent reticulocytes. The serum bilirubin values were 1.4 mg direct and 3.9 mg indirect. A fragility test showed initial hemolysis at 0.66 per cent and complete hemolysis at 0.36 per cent. The bromsulfalein test for liver function did not disclose any dye retention. During the first forty-eight hours in the hospital, the average amount of fecal urobilinogen excreted each twenty-four hours was 4,800 mg, during the next forty-eight hours, this averaged 1,220 mg. No irregular agglutinins were demonstrable.

The diabetes was carefully controlled. Splenectomy was performed on December 13. The spleen



weighed 1,570 Gm. The postoperative convalescence was uneventful and no transfusion of blood administered. The amounts of urobilinogen excreted in the feces were as follows: on December 17 and 230 mg. per twenty-four hours, on December 19 and 20, 85 mg. per twenty-four hours, on December 22, 45.5 mg. per twenty-four hours.

On December 23, the value for the indirect serum bilirubin was 0.6 mg. When the patient was dismissed on January 4, 1947, the hemoglobin value was 8.7 Gm. and the erythrocytes numbered 2,900 c.

In a letter dated March 17, 1947, the patient stated that her blood picture had improved. The hemoglobin value was 13.6 Gm., the erythrocyte count was 3,760,000 and the leukocyte count was 11,600.

In case 9, which belongs to the microspherocytic group, the result has been excellent. In this group, the results of splenectomy as a whole were considered good. In 9 of the 11 cases, the patients were women. There was one death in this group and in one case (case 3) the patient was not improved. In case 11 the patient was improved but did not obtain an excellent result.

*Case 13* A boy, aged 11, came to the clinic July 31, 1942. He had had intermittent attacks of jaundice since the age of 2, when he had had a febrile illness which had been diagnosed as Malta fever. The ordinary contagious diseases of childhood, such as whooping cough, measles and scarlet fever, each had been followed by jaundice. The jaundice also had occurred after an infection of the upper part of the respiratory tract.

When the patient was examined at the clinic, he was slightly icteric and the spleen could be palpated 2 inches below the costal margin. The hemoglobin value was 10.5 Gm., the erythrocyte count was 3,270,000 with 10 per cent reticulocytes, and the leukocyte count was 5,400. Examination of a blood smear disclosed active regeneration of the erythrocytes but no microspherocytosis. The indirect serum bilirubin value was 2.2 mg. and the bromsulfalein test for liver function disclosed no dye retention. The fragility of the erythrocytes was normal. It was felt that the patient had hemolytic anemia of an acquired type. Splenectomy was advised.

The patient returned to the clinic May 25, 1944. In August, 1943, he had had an attack of epigastric pain. He had been markedly jaundiced and his temperature had reached 103°F. After ten days he had had a rapid recovery. Examination disclosed hematologic findings as they were at the time of the patient's first visit to the clinic with the exception that the indirect serum bilirubin value was 5.5 mg. Splenectomy was performed on May 29. The spleen weighed 266 Gm. When the patient was dismissed, the value for the hemoglobin and the erythrocyte and leukocyte counts were the same as they had been when the patient came to the clinic but the indirect serum bilirubin value had dropped to 0.5 mg.

He returned to the clinic July 19, 1944, because of an attack of pain in the upper part of the abdomen and jaundice. At this time, the hemoglobin value was 12.3 Gm., the erythrocyte count was 3,880,000 and the leukocyte count was 11,800. Microcytosis of the erythrocytes was observed for the first time. No change in the hematologic findings was observed when the patient was seen again on August 8, 1946. Attacks of jaundice had continued to occur. The patient's growth has continued in an apparently normal manner.

*Case 15* A married woman, aged 21, came to the clinic April 20, 1945. In January, 1944, she had had a miscarriage at the fourth month of pregnancy. During the pregnancy, her parents had thought that she had appeared yellow. After the miscarriage, she had lost weight and had become very weak. She had been treated for anemia. There was no family history of hemolytic anemia. Serologic tests for syphilis on both the blood and spinal fluid had been strongly positive, and antisyphilitic treatment had been administered intramuscularly. There was no personal or family history of syphilis and the patient denied the possibility of contact infection. Between March 27 and April 12, 1945, she was given a total of 2,500 cc. of blood in nine transfusions. She had been told that her hemoglobin value was lower after these transfusions than it had been previously.

When she was examined at the clinic, the hemoglobin value was 8.8 Gm., the erythrocyte count was 2,520,000 and the leukocyte count was 8,200. Examination of a blood smear revealed a marked increase in the regeneration of the erythrocytes and a regenerative macrocytosis. There was abundant myeloid

immaturity but no evidence of microspherocytosis. The indirect serum bilirubin value was 1.6 mg. The fragility test revealed that hemolysis began at 0.44 per cent and was complete at 0.34 per cent. The brom-sulfalein test disclosed no dye retention. The Kline, Kahn, Hinton and Kolmer serologic tests for syphilis were negative.

On May 2, the hemoglobin value was 4.5 Gm, the erythrocyte count was 1,470,000 with 32.2 per cent reticulocytes, and the leukocyte count was 7,500. The amounts of urobilinogen excreted in the feces were as follows: On May 1 and 2, 894 mg per twenty-four hours, on May 3 and 4, 642 mg per twenty-four hours. A transfusion of 500 cc of blood was administered on three occasions between May 3 and May 9. Splenectomy was performed on May 9. The spleen weighed 670 Gm. Another transfusion of blood was given on May 21.

The patient was greatly improved when she returned to the clinic for examination on October 24, 1945. The hemoglobin value was 12.7 Gm, the erythrocyte count was 4,620,000 with 8.6 per cent reticulocytes and, the leukocyte count was 12,900. In the blood smear there was active regeneration of the erythrocytes with many macrocytes. The amount of urobilinogen excreted in the feces was determined for a period of four days. The average amount was 147 mg per twenty-four hours. The indirect serum bilirubin value was 0.45 mg.

On March 25, 1947, the patient's family physician informed us that the hemoglobin value was 78 per cent and that the erythrocyte count was 4,120,000.

In the group of cases without significant microspherocytosis, females again predominated. The higher incidence of this disease among females has also been noted by Fowler. In this group, the results have not been as good as they were in the microspherocytic group although the number of cases is not large enough to draw definite conclusions. However, the results are encouraging enough to warrant further trial of splenectomy. A longer period of observation is desirable to determine how frequently hemolytic episodes may occur after operation.

#### COMMENT

In recent years several excellent reviews dealing with hemolytic anemia have appeared.<sup>1, 2, 7</sup> Watson has classified the hemolytic anemias as microcytic (familial or congenital) and macrocytic (secondary or acquired). He stated that in all cases of the acquired type of the disease the erythrocytes are at least slightly larger and often much larger than the normal. Dameshek and Schwartz and Singer and Dameshek, pointed out that in some cases of acquired hemolytic anemia, spherocytosis and increased hypotonic fragility are present, although a "pseudomacrocytic" blood picture may be seen. Fowler found that spherocytosis was not consistently present in a group of cases of acquired hemolytic anemia and that macrocytosis was more frequently encountered.

All of our cases were examples of primary nonfamilial hemolytic anemia so far as we could determine. Microspherocytosis was not present in half of these cases but with one exception (case 13) we could not classify them as cases of macrocytic anemia. There was a considerable number of macrocytes in some of the smears but many of them were regenerative or polychromatophilic erythrocytes.

Agglutinins and hemolysins may be etiologic factors in a hemolytic syndrome. In two of our cases (cases 14 and 22) iso-agglutinins of an abnormal type were present. In each instance, the patient's serum agglutinated his own erythrocytes. In another case (case 4), an Rh negative patient had a high Rh antibody titer due to previous transfusions of Rh positive blood. Although the blood picture was micro-

spherocytic, it is possible that this antibody titer may have been the cause of hemolytic anemia. In any event, improvement did not occur until splenectomy done. At the present time, a more intensive search for irregular agglutinins hemolysins is being carried out in certain cases of hemolytic anemia.<sup>5</sup>

Several authors have emphasized the dangers of severe hemolytic reactions following blood transfusion in hemolytic anemia. In one of our cases (case 10), death was probably due to a hemolytic transfusion reaction after operation. We have noted any severe exacerbation of the hemolytic process in the other cases but have been impressed with the failure of transfusion to benefit the patient, especially before splenectomy.

We have found it difficult to correlate the degree of anemia with the severity of the jaundice. In one case (case 4) as long as the patient was severely jaundiced the anemia was relatively mild. When the severity of the jaundice decreased, the concentration of hemoglobin decreased rapidly. This inverse relationship has been noted by Watson and Fowler.

In several of our cases bone marrow was examined. A definite hyperplasia of normoblastic cells was seen in each instance. No megaloblasts were found.

Although not common, leukopenia and thrombocytopenia may accompany hemolytic anemia. Doan and Wright have recently reported this phenomenon as a pancytopenia. In case 14, the number of leukocytes ranged from 3,100 to 5,000 and the number of thrombocytes from 65,000 to 75,000 per cubic millimeter before splenectomy. Both were normal or increased in number after operation.

In a case not included in this series splenectomy was performed for what appeared to be a primary hemolytic anemia. The blood picture was subsequently that of chronic myelogenous leukemia. At the time of the original examination, there was not as much myeloid immaturity as there was in the blood of many of the patients in the present series. The sternal marrow was hyperplastic and could not be distinguished from nonleukemic hyperplastic marrow.

Splenectomy may be of definite benefit in symptomatic hemolytic anemia when the progress of the primary disease is not rapid. Recently, a woman who was 60 years of age came to the clinic because of weakness of six months' duration. The hemoglobin value was 8.3 Gm, the erythrocyte count was 2,250,000 with 15 per cent reticulocytes. A spleen which weighed 1,125 Gm was removed and a diagnosis of follicular lymphoblastoma was made. There were no enlarged lymph nodes. One year later, the patient, who had regained her good health, returned because of enlarged axillary and inguinal lymph nodes. The hemoglobin value then was 11 Gm and the erythrocyte count was 4,150,000. Biopsy of a lymph node confirmed the previous diagnosis and for the first time roentgen therapy was started. The splenectomy had relieved the weakness and anemia.

#### SUMMARY

In our experience, in half of the cases of primary hemolytic anemia in which there is no family history of anemia, jaundice or splenomegaly, examination of the blood disclosed microspherocytic erythrocytes and increased fragility of erythrocytes. The results of splenectomy in these cases are better than in those in which

microspherocytosis is absent True macrocytosis was observed in only one instance Females predominated in both groups of cases Agglutinins and hemolysins have not appeared to play any significant role in the production of the hemolytic syndrome in our cases We do not feel justified in expressing an opinion as to whether the microspherocytosis indicates a familial or congenital blood disorder From a practical standpoint, it makes no great difference since splenectomy should be considered seriously in any case of chronic primary hemolytic anemia It may be of value in some cases of secondary or symptomatic hemolytic anemia

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# THE GENETIC RELATION AND CLINICAL DIFFERENTIATION OF COOLEY'S ANEMIA AND COOLEY'S TRAIT

By GENEVA A. DALAND, B.S., AND MAURICE B. STRAUSS, M.D.

IN 1925<sup>1</sup> the late Thomas B. Cooley first described the severe and generally fatal anemia that is now variously known as Cooley's Anemia, Mediterranean anemia and thalassemia. Since then numerous workers<sup>1-10</sup> have reported cases of a mild blood disorder which resembles Cooley's anemia but is not associated with the symptoms or disability. This disorder, found almost exclusively in Italian and Greek families, is characterized by microcytosis and hypochromia with marked variation in the size and shape of the erythrocytes and by increased red cell resistance to lysis in hypotonic salt solution. The hemoglobin values are either normal or moderately reduced while the red blood cells are generally increased in number. For reasons to be discussed later the term "Cooley's Trait" is proposed for this disorder and will be used throughout this paper. Neel and Valentine<sup>11</sup> have estimated that this condition is present in 4 per cent of persons of Italian ancestry in Rochester, N. Y., and one of us has noted it frequently as an incidental finding in soldiers of Mediterranean ancestry in an Army General Hospital.

Since earlier publication,<sup>5</sup> 4 additional families have been studied, one family including 3 cases of Cooley's anemia. One of the patients with Cooley's anemia has given birth to a living child, an event thus far unreported. This child exhibits Cooley's trait.

It is the purpose of this publication to present the hematologic and genetic data obtained from the examination of these families, to clarify further the inheritance of both Cooley's trait and Cooley's anemia and to emphasize the clinical and hematologic differentiation of the two conditions.

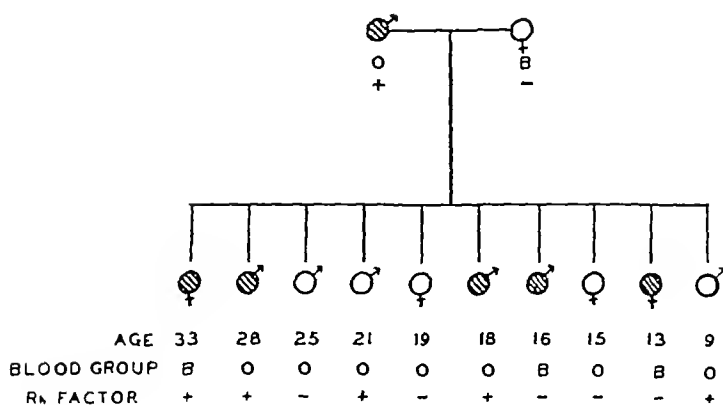
## OBSERVATIONS

*Family Ca* A 16 year old boy of Italian parentage came under observation during an acute attack of rheumatic fever. He was found to have a hypochromic microcytic anemia with marked variation in cell size and shape and increased red cell resistance to lysis by hypotonic salt solution. Twelve grains of ferrous sulfate daily for one month failed to alter his blood values. Blood studies on the patient, his father, mother and 9 siblings are recorded in table 1 and figure 1. All the members of the family were healthy and completely asymptomatic. No member was known to have been anemic or seriously ill.

*Family Cr* A 32 year old woman first came under observation after she had been unsuccessfully treated for a mild anemia for several years. Her blood study exhibited all the characteristics of Cooley's trait. Accordingly other members of the family were examined. The data are shown in table 2 and figure 2. Both parents had been born near Naples, Italy. The father had died many years before. The mother died of pneumonia in 1941. In 1938 she had been observed to have undue variation in the size and shape of her erythrocytes and considerable basophilic stippling. When she had pneumonia she was anemic and nucleated red blood cells were observed in stained blood films.

*Family Sp* This family was reported in detail from this laboratory by Spodaro and Forkner<sup>2</sup> in 1933.

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FIG 1 *Family Ca*TABLE I—*Blood Findings in the Ca Family*

	Mr C	Mrs C	Ca	Jo	Ja	Pe	Ph	Ay	Fr	Ma	An	Ro
Age			33	28	25	21	19	18	16	15	13	9
Sex	M	F	F	M	M	M	F	M	M	F	F	M
<i>Blood examination</i>												
R B C, M per cu mm	5 6	4 8	5 5	6 0	4 9	4 5*	4 1*	5 8*	5 8	4 4*	5 6	4 4
Hb, %	75	76	71	81	90	92	79	75	66	84	66	82
Hb, Gm per 100 cc blood	11 7	11 8	11 1	12 6	14 0	14 4	12 3	11 7	—	13 1	10 3	12 8
Hematocrit, %	40 3	39 1	38 0	39 8	43 3	42 6	38 6	35 2	35 4	40 2	38 2	40 1
M C V, cu $\mu$	72 2	81 1	69 8	66 8	88 7	94 6	94 4	60 9	60 8	91 8	68 4	90 7
M C H concentr, %	29 0	30 3	29 0	31 8	32 4	33 7	31 9	33 2	29 1	32 6	27 0	31 9
M C H, micromicrograms	21 0	24 6	20 4	21 2	28 8	31 9	30 1	20 2	17 7	29 9	18 4	28 9
Reticulocytes	1 1	6	3 1	1 0	0 5	0 8	1 3	0 8	2 6	0 8	1 0	1 5
Nucleated red cells	0	0	0	0	0	0	0	0	0	0	0	0
Stippling	0	0	0	+	0	0	0	+	+	0	0	0
Icteric index	—	5	—	—	5	7	—	—	4	5	5	—
W B C, thousands per cu mm	8 2	7 8	6 8	7 9	6 5	5 3	11 5	8 3	10 3	7 4	6 8	5 6
Blood group	O	B	B	O	O	O	O	O	B	O	B	O
Blood type	N	N	N	N	N	N	N	N	N	N	N	N
Rh factor	+	—	+	+	—	+	—	+	—	—	—	+
Blood smear	Abn	N	Abn	Abn	N	N	Abn	Abn	Abn	Abn	Abn	N

\* Counts taken following satisfactory therapy with iron

N, normal, Abn, abnormal

Red Cell Resistance—Per cent hemolysis in hypotonic solutions of sodium chloride

	1%	10%	50%	75%
Francis	42	37	32	29
Normal	43	38	35	33

under the title Benign Familial Polycythemia, but since it is now believed that this condition is identical to Cooley's trait the essential data are presented in table 3 and figure 3

*Family Pa* The oldest daughter of this family was first observed at the Massachusetts General Hospital in 1934, when she was 19 years of age. At this time she had acute pyelonephritis and a diagnosis was

discovered to have the characteristic blood picture of Cooley's anemia, as well as jaundice, splenomegaly and changes in the bones. She maintained approximately the same blood level for the next five years but died with severe anemia a fortnight after the birth of a child. At the time of delivery her red blood count was reported to be 1.12 million per cu mm and the hemoglobin 3.9 Gm per 100 cc.

In 1934, when the diagnosis of Cooley's anemia was first made on the above patient, her parents and 5 siblings were examined. At that time a brother, aged 7, and the youngest sister, aged 16, were considered to have Cooley's anemia, but the mother, father and 3 other sisters were believed to be normal. On subsequent examination by the authors, however, all but one sister were found to have Cooley's trait (table 4, fig. 4). The brother had many hospital admissions and finally died at the age of 11. His autopsy findings were typical of Cooley's anemia.

The youngest sister has been followed in the Thorndike Memorial Laboratory since 1938, during which time she has continued to present a characteristic picture of Cooley's anemia. In March 1946 she was delivered of a male infant whose blood has been examined on several occasions. On October 1946 his red cells numbered 5,330,000 per cu mm and his hemoglobin was 9.4 Gm per 100 cc. Following treatment with iron for two months the red cell count was 6,100,000 and the hemoglobin 14.0 G. His blood films are definitely abnormal, showing variation in size of the red cells, variation in intensity

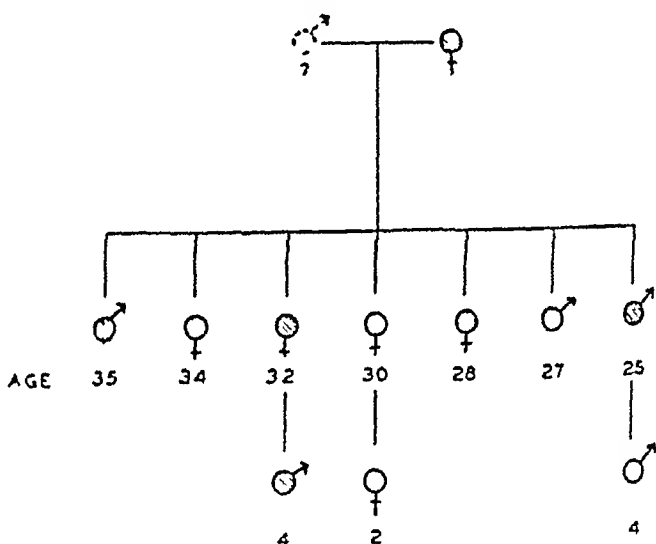


FIG. 2. Family Cr

of staining, microcytes, occasional pencil forms and fragmented forms. No stippled cells were seen. This picture has been maintained throughout the observations and is a distinctly unusual picture when the red count and hemoglobin are so high. With the exception of frequent colds, he has been entirely well. His father has an entirely normal blood (table 4).

#### LITERATURE

During 1940 and 1941, three groups of investigators independently described the mild blood disorder illustrated by the cases presented above. Wintrobe and his co-workers<sup>3</sup> considered the condition to be a benign form of Cooley's anemia. Dameshek<sup>4</sup> described it as "target cell" anemia. These authors, both then and in later publications,<sup>9, 14</sup> emphasized the existence of a continuous series of conditions from the very mild cases of Cooley's trait to the most severe Cooley's anemia. The present authors<sup>5</sup> termed the mild disorder "Familial Microcytic Anemia," stressing the fact that although it resembled Cooley's anemia, clinical and hematologic differentiation was not difficult. It was further stated that the mild disorder

TABLE 2 — *Blood Findings in the Cr Family*

TABLE 2 — Blood Findings in the Cr Family												
Age	Sex	Mrs Cr	Children							Grandchildren		
			M <sub>1</sub>	Lo	M T	L D	J C	Pa	An I	F T	S D	An II
			35	34	32	30	28	27	25	4	2	4 days
Blood examination												
R B C, M per cu mm		4 7	5 7	4 6	4 8	5 4	4 4	5 7	6 4	6 2	5 3	6 3
Hb, %		95	100	87	69	87	66	101	83	84	92	123
Hb, Gm per 100 cc blood		14 8	15 6	13 5	10 7	13 6	10 3	15 7	12 9	13 1	14 3	19 2
Hematocrit, %		42 4	46 0	38 7	32 3	40 0	30 3	46 4	39 7	39 4	63 5	—
M C V, cu $\mu$		90 2	82 1	84 1	67 5	75 4	70 4	81 4	62 0	63 5	—	—
M C H concentr, %		34 9	33 9	34 0	33 3	33 9	33 9	33 5	32 6	33 2	—	—
M C H, micrograms		31 5	27 8	29 5	22 4	35 6	23 9	27 6	20 2	21 1	—	—
Reticulocytes, %		—	2 6	1 7	2 9	1 3	3 4	1 2	1 4	2 3	—	—
Nucleated red cells		0	0	0	0	0	0	0	0	0	0	0
Stippling		+++	+	+	+	+	+	+	+	+	+	+
Icteric Index		—	6	5	4	5	5	5	7 5	6	—	—
Red cell resistance		—	—	—	—	—	—	—	—	—	—	—
Trace of hemolysis		—	—	—	—	—	—	—	—	—	—	—
Complete hemolysis		47	46	46	42	46	—	46	44	42	—	—
Platelets, thousands per cu mm		32	29	29	17	29	—	27	18	17	—	—
White blood cells, thousands per cu mm		—	270	286	244	300	229	286	174	300	—	244
Polymorphonuclear neutrophils, %		9 8	6 6	6 0	8 0	9 0	10 5	6 9	5 0	10 0	9 1	9 5
Polymorphonuclear eosinophils, %		55 0	44 0	55 0	72 0	55 0	70 0	44 0	45 0	50 5	25 0	46 5
Polymorphonuclear basophils, %		7 0	2 0	1 0	2 0	3 0	3 0	3 0	2 0	3 0	0	6 5
Small lymphocytes, %		0	0	1 0	1 0	3 0	1 0	1 0	0	0 5	0	0 5
Large lymphocytes, %		30 0	45 0	39 0	21 0	36 0	20 0	45 0	17 0	38 0	70 0	8 0
Monocytes, %		0	0	0	0	0	0	0	0	0	0	0
Abnormal young cells, %		8 0	9 0	4 0	4 0	3 0	6 0	7 0	34 0	0	0	35 5
Myelocytes, %		0	0	0	0	0	0	0	0	0	0	0
Blood smear		Abn	N	N	Abn	N	Abn	Abn	Abn	Abn	Abn	Abn
N, normal, Abn, abnormal												

N, normal, Abn, abnormal



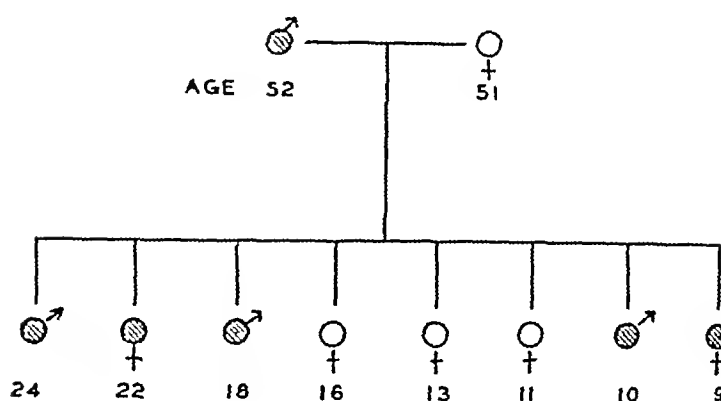


FIG 3 Family Sp

TABLE 3—Blood Findings in Sp Family (Ref 2)

Age	52	51	24	22	18	16	13	11	10
Sex	M	F	M	F	M	F	F	F	M
<i>Blood examination</i>									
RBC, M per cu mm	6 8	5 5	8 0	7 1	8 1	5 0	5 6	4 9	6 4
Hb, Gm per 100 cc blood	13 6	14 6	15 8	12 7	14 5	12 3	15 4	12 5	12 5
Hematocrit %	50 3	46 7	50 0	49 5	57 0	—	46 9	45 2	42 6
M C V, cu $\mu$	74 2	85 6	63 2	69 7	70 6	—	83 5	92 6	66 2
M C H, concentr, %	27 0	31 2	31 5	25 7	25 5	—	32 8	27 6	29 3
M C H, micromicrograms	20 0	26 7	19 7	17 9	18 0	—	27 3	25 6	19 4
Reticulocytes, %	2 4	1 6	—	2 2	1 0	0 6	1 2	1 4	2 4
WBC, thousands per cu mm	8 1	6 9	8 5	7 0	8 8	7 3	11 4	8 3	8 0
Polymorphonuclear neutrophils, %	63 0	66 0	63 0	63 0	57 0	58 0	56 0	64 0	64 0
Polymorphonuclear eosinophils, %	2 0	1 0	—	2 0	4 0	1 0	2 0	1 0	3 0
Polymorphonuclear basophils, %	—	1 0	4 0	1 0	2 0	3 0	3 0	2 0	1 0
Small lymphocytes, %	28 0	28 0	21 0	31 0	21 0	31 0	31 0	25 0	22 0
Monocytes, %	7 0	4 0	12 0	3 0	16 0	7 0	8 0	8 0	10 0
Platelets	Normal	Normal	+	+	Normal	Normal	+	—	+

appeared to be inherited as a dominant character whereas Cooley's anemia was believed to be recessive<sup>15 16</sup> Although Angelini,<sup>17</sup> and independently Caminopetros,<sup>15</sup> had observed that the parents and siblings of patients with Cooley's

anemia frequently exhibited increased red cell resistance to lysis by hypotonic salt solution, it was not until Wintrobe<sup>14\*</sup> pointed out that in two different patients with Cooley's anemia *both* parents exhibited the mild blood disorder, that the genetic relationship between the two conditions became apparent. Wintrobe's observations were confirmed\* by Smith,<sup>7, 8</sup> Dameshek<sup>9</sup> and Valentine and Neel,<sup>10</sup> who in 1944 published a thorough discussion of the subject and added data from four additional families.

#### DIFFERENTIATION OF COOLEY'S TRAIT FROM COOLEY'S ANEMIA

Although it is possible by proper selection of cases to show a continuous series of conditions grading from the most severe type of Cooley's anemia fatal in early life to the most mild instances of Cooley's trait in which there is no anemia whatsoever, it is our impression that generally the differentiation of the two conditions is not difficult although both are characterized by hypochromia, microcytosis,

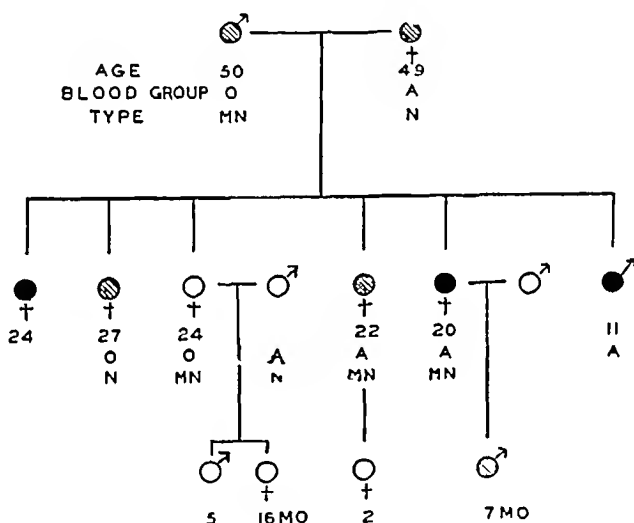


FIG 4 Family Pa

anisocytosis and poikilocytosis out of proportion to the hemoglobin levels. The blood pictures fall into two fairly well defined groups, one severe and generally fatal, the other benign. † Correlated with these two blood pictures may be found marked clinical signs and symptoms in the case of Cooley's anemia, or their absence or minor nature, in Cooley's trait. The differential diagnostic findings between Cooley's anemia and Cooley's trait are shown in table 5.

\* Although the matter of priority as regards this particular point is of little significance, it should be pointed out that Dameshek (Hemolytic Syndromes Exhibit American Medical Association, June 1942. Privately printed 1942, and Bull. New England Med. Centre 467, 1942) also clearly pointed out this hereditary mechanism. These observations were made independently and were therefore not confirmatory, as stated Editor.

† Attempts to divide this entire disease syndrome into two groups, one severe and one benign, are not always successful, since a number of intermediate forms with moderate anemia, splenomegaly, and icterus are found. It seems hardly desirable to designate cases of well-defined anemia as trait, especially since various symptoms referable to the anemia are present. F<sub>2</sub>/cr

## GENETIC CONSIDERATIONS

Three main types of inheritance could explain the relationship between Cooley's anemia and Cooley's trait<sup>10</sup>

1 *A single dominant factor variably expressed* An example of this type of inheritance is found in the variable severity of hemophilia which the sons of one mother may exhibit, each of whom owes his disease to an identical gene. If this

TABLE 4—Blood Findings in the Pa Family

	Mr P	Mrs P	M P S	J P B
Age	50	49	24	26
Sex	M	F	F	F
<i>Blood examination</i>				
R B C, M per cu mm	5 6	6 0	3 6	5 0
Hb, %	86	92	52	84
Hb, Gm per 100 cc blood	13 4	14 3	8 1	13 1
Hematocrit, %	43 9	44 5	27 4	41 1
M C V, cu $\mu$	77 3	74 4	76 1	82 8
M C H concentr, %	30 6	32 2	29 6	32 0
M C H, micromicrograms	23 6	24 0	22 5	26 5
Reticulocytes, %	0 6	0 7	11 8	0 7
Nucleated red cells	0	0	+	+
Stippling	++	+	+	+
Icteric index	4	4	—	3
Red cell resistance*				
		% Hem NaCl	Hem NaCl	% Hem NaCl
		1 49	Trace 58	1 46
		10 41	Complete 16	10 40
		50 37		50 34
		75 34		75 32
Platelets, thousands per cu mm	+	N	+	N
White blood cells, thousands per cu mm	5 9	8 8	12 4	6 8
Polymorphonuclear neutrophils, %	57 0	49	55	48
Polymorphonuclear eosinophils, %	3	3	1	9
Polymorphonuclear basophils, %	—	—	—	2
Small lymphocytes, %	25	27	18	18
Large lymphocytes, %	9	11	19	5
Monocytes, %	4	8	5	5
Abnormal or young cells, %	1	1	—	3
Myelocytes, %	1	1	2	—
Blood group	O	A	—	N
Blood type	MN	N	—	+
Rh factor	+	+	—	Abn
Blood Smear	Abn	Abn	Abn	

TABLE 4—Continued

	J P C	A P C	A P T	M P
Age	23	22	20	11
Sex	F	F	F	M
<i>Blood examination</i>				
R B C, M per cu mm	4 8	5 0	5 1	2 5
Hb, %	105	76	62	28
Hb, Gm per 100 cc blood	16 4	11 8	9 7	4 4
Hematocrit, %	44 6	40 6	29 0	
M C V, cu $\mu$	92 3	80 2	56 9	
M C H concentr, %	36 7	29 2	30 0	
M C H, micromicrograms	33 9	23 4	19 0	
Reticulocytes, %	0 8	1 5	2 8	6 2
Nucleated red cells	0	0	+	+++
Suppling	0	++	++	++
Icteric index	4	5	10	
Red cell resistance*				
	% Hem NaCl	% Hem NaCl	% Hem NaCl	
	1 46	1 44	1 49	
	10 41	10 39	10 43	
	50 37	50 35	50 28	
	75 35	75 33	75 23	
Platelets, thousands per cu mm	N	N	264	+
White blood cells, thousands per cu mm	11 8	8 6	11 2	4 4
Polymorphonuclear neutrophils, %	60	7-	70	56
Polymorphonuclear eosinophils, %	2	2	1	-
Polymorphonuclear basophils, %	1	-	-	
Small lymphocytes, %	20	1-	1-	25
Large lymphocytes, %	10	-	6	-
Monocytes, %	-	9	-	15
Abnormal or young cells, %	-	-	1	-
Myelocytes, %	-	-	1	-
Blood group	O	A	A	A
Blood type	MN	MN	MN	-
Rh factor	-	-	-	-
Blood Smear	N	Abn	Abn	Abn

TABLE 4—*Concluded*

	Family of J P C			Family of A P C	Family of A P T	
	Mr C	P C	K C	J C	Mr T	D T
Age	33	5 yr	16 months	2	—	months
Sex	M	M	F	F	M	M
<i>Blood examination</i>						
R B C, M per cu mm	55	48	50	—	56	53
Hb, %	108	85	86	85	116	60
Hb, Gm per 100 cc blood	16.9	13.2	13.4	—	18.0	9.3
Hematocrit, %	49.5	38.2	—	—	50.2	—
M C V, cu $\mu$	90.0	80.2	—	—	90.0	—
M C H concentr, %	34.1	34.6	—	—	35.8	—
M C H, micromicrograms	30.7	27.7	26.9	—	32.2	17.6
Reticulocytes, %	1.6	1.1	0.6	—	—	—
Nucleated red cells	0	0	0	—	—	0
Stuppling	0	0	0	—	—	0
Icteric index	5	5	—	—	7	—
Platelets	N	N	N	—	N	N
W B C, thousands per cu mm	6.7	9.7	—	—	8.7	9.3
Blood Group	A	O	—	—	—	—
Blood Type	N	MN	—	—	—	—
Rh factor	—	+	—	—	—	—
Blood Smear	N	N	N	N	N	Abn

K C and J C show a few young cells which are to be expected at their ages. R B C are normal. D T showed more variation in size and shape than normal. Cells were somewhat hypochromic.

TABLE 5—*Clinical and Hematologic Differentiation of Cooley's Anemia and Cooley's Trait*

	Cooley's Anemia	Cooley's Trait
Red Blood Cell Count	<5,000,000	5,000,000–8,000,000
Mean Corpuscular Volume	50 to 100 cu micra	55 to 80 cu micra
Hemoglobin	<10 Gm per 100 cc	9 to 16 Gm per 100 cc
Nucleated Red Blood Cells	Common	Rare
Reticulocytes	Increased	<4 per cent
Polychromatophilic Cells	Increased	Occasional
Stuppled Cells	Marked	Frequent
Icteric Index	Increased	Normal
Leukocytes	Increased	Normal
Poikilocytosis	Extreme	Moderate
Bone Changes	Generally in skull and other bones	Skull only (slight)
Splenomegaly	Common	Occasional
Features	Mongoloid	Normal
Age	Young	Any
Prognosis	Poor	Excellent

mode of inheritance were involved, Cooley's anemia should appear in families in which only one parent exhibited a blood abnormality. However, in essentially every case in which both parents of a patient with Cooley's anemia have been

examined, both have shown the mild disorder. Since, in accord with this theory, only one parent need be affected, and since marriages between two affected individuals should be much rarer than between one affected and one normal person, most cases of Cooley's anemia would be the issue of the latter type of union.

2. *The simultaneous presence of two nonallelomorphic dominant factors, one inherited from each parent*, as suggested by McIntosh and Wood,<sup>18</sup> would result in Cooley's anemia. This theory requires the assumption that each factor alone leads to the same mild somatic condition, which Valentine and Neel<sup>10</sup> consider improbable. However, it may be noted that in *Drosophila* a number of different factors may lead to the identical eye color. There are other more complex types of inheritance which may be considered in which two nonallelomorphic factors are required for the development of the full-blown character and in which heterozygosity leads to a partial abnormality. For example, "ski-wing" in *Drosophila* results from homozygosity of two genes in the second and third chromosomes. If the gene in the second chromosome is heterozygous and the gene in the third chromosome homozygous, a mild form of ski-wing results.

3. *A single "incomplete" recessive or dominant factor*. Under this type of inheritance the heterozygote would exhibit Cooley's trait and the homozygote, Cooley's anemia.

The data presented here, together with the extensive statistical analysis of Valentine and Neel<sup>10, 19</sup> are compatible with either the second or the third mode of inheritance.

#### NOMENCLATURE

Spodaro and Forkner<sup>2</sup> termed this mild blood disorder "benign familial polycythemia." Since polycythemia is not necessarily present this designation is unsuitable. The present authors have called it "familial microcytic anemia,"<sup>5</sup> a name which must be rejected since anemia may be absent.

Wintrobe et al.<sup>2</sup> called this condition "a familial hemopoietic disorder in Italian adolescents and adults resembling Mediterranean disease (thalassemia)." Dameshek<sup>4</sup> referred to it as "target cell anemia." However, target cells are encountered in many conditions and may be artificially produced in vitro by suspending normal cells in plasma or serum rendered hypertonic.<sup>20</sup> Later Dameshek used the designation "familial Mediterranean target-oval cell syndromes."<sup>9</sup> Since cases have been reported in English, Negroes, Chinese, Turkish, Spanish and others<sup>21</sup> and since there is a distinct blood disorder characterized by oval erythrocytes<sup>22</sup> this name does not appear suitable. Valentine and Neel's<sup>10</sup> terminology, "thalassemia major and thalassemia minor" for the severe and mild disorders, has much in its favor. However, the wide distribution of these conditions, even if their common origin at one time was the Mediterranean and the cumbersome terms have led the present authors to call the conditions "Cooley's anemia" and "Cooley's trait," bearing in mind the analogous "sickle cell anemia" and "sickle cell trait." It is not implied that sickle cell anemia and sickle cell trait bear the same genetic relation as Cooley's anemia and Cooley's trait. Although eponymic nomenclature is in general undesirable, it may be pointed out that a number of syndromes such as Graves' disease, which may occur without goiter, without hypothyroidism, without ophthalmopathy, defy accurate descriptive terminology.

## SUMMARY

- 1 Four additional families illustrating the clinical and genetic relationships of Cooley's anemia and Cooley's trait have been presented
- 2 Blood findings in an offspring of a patient with Cooley's anemia are recorded
- 3 The asymptomatic nature of Cooley's trait and its differentiation from Cooley's anemia has been emphasized
- 4 The inheritance of Cooley's trait and Cooley's anemia may be best explained in terms of an incomplete dominant or of the simultaneous appearance of two nonallelomorphic genes

## ACKNOWLEDGMENT

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## STUDY OF THALASSEMIA MINOR IN THREE GENERATIONS OF AN ITALIAN FAMILY

By ROBERT W HEINLE, M D , AND MARGARET RUTH READ, M D

A CONSIDERABLE knowledge concerning the heredity and transmission of Cooley's erythroblastic anemia, or thalassemia, has accumulated during the past twenty years but there is still need for more complete data and further genetic studies of families showing the trait

In 1927, Cooley<sup>1</sup> reported his observations on erythroblastic anemia occurring in Mediterranean peoples. He concluded that the anemia was congenital, but in spite of frequent familial occurrence, he doubted that heredity was a factor since the patients died before puberty and so could not transmit the disease

It was recognized for the first time in 1937 that a mild, but similar, type of anemia occurred in parents and siblings of individuals with Cooley's anemia. Angelini (quoted by Wintrobe and his associates<sup>2</sup>) observed that in some instances, the erythrocytes of apparently healthy parents and siblings of patients with Cooley's anemia showed decreased fragility when tested in hypotonic saline. Caminopetros<sup>3</sup> independently confirmed this observation in 1938

Two years later, Wintrobe and co-workers,<sup>2</sup> Dameshek<sup>4</sup> and Strauss and co-workers<sup>5</sup> described a mild form of microcytic hypochromic anemia, resistant to iron therapy, and occurring in Italian families. The anemia was characterized by the frequent occurrence of increased numbers of erythrocytes, with absolute and relative reduction of hemoglobin, bizarre forms of erythrocytes and decreased fragility in hypotonic saline. These authors variously termed the dyscrasia as 'target cell anemia,' "familial microcytic anemia" and 'anemia of adults resembling thalassemia'. It was not immediately apparent whether this disease was related genetically to Cooley's Mediterranean anemia, but such a possibility was suggested

Wintrobe<sup>6</sup> later confirmed Angelini's and Caminopetros' observation of decreased fragility of erythrocytes in the parents of patients having Cooley's erythroblastic anemia and further pointed out that the blood picture in the parents was identical with the familial microcytic hypochromic anemia which he had previously described.<sup>2</sup>

In 1942 and 1943, Dameshek<sup>7</sup> described several anemic states of varying degree of severity ranging from Cooley's erythroblastic anemia to conditions with mild hypochromic anemia, target, oval and stippled cells and increased resistance of the erythrocyte to hypotonic saline. He confirmed the findings of other workers in demonstrating that such blood changes occurred in both siblings and parents of patients with thalassemia. Smith<sup>8</sup> also demonstrated similar changes in the blood of siblings of patients with Cooley's anemia and discussed the diagnosis of the "trait" or mild form of the disease



Valentine and Neel,<sup>9</sup> in reporting studies on parents and siblings of 3 patients with thalassemia and of one person with a similar mild condition, considered the hereditary aspects of the disorder and emphasized the problem of differential diagnosis and the clinical significance of the mild form of the anemia. By statistical analysis of cases collected from the literature, they concluded that the bulk of evidence favored the hypothesis that the mild, microcytic hypochromic anemia was a form of thalassemia which resulted from heterozygosity for a factor which, when homozygous, caused full-blown thalassemia. These authors suggested the term "thalassemia major" for the severe erythroblastic anemias as originally described by Cooley and "thalassemia minor" for the mild microcytic hypochromic anemias characterized by target cells, oval cells and increased resistance of the erythrocytes to hypotonic saline.

Cooley<sup>10</sup> has more recently described a microcytic hypochromic anemia occurring only in males with transmission through the females in a family of Dutch descent. His conclusion that the disease was due to a fundamental constitutional defect of the hematopoietic system unrelated to familial microcytic anemia may be open to some doubt.

In 1945, Neel and Valentine<sup>11</sup> reiterated their views regarding "thalassemia major and minor" and the inheritance of the conditions. From a study of a portion of the Italian population of Rochester, N. Y., they concluded that thalassemia major occurred in about 0.042 per cent and thalassemia minor in about 4 per cent.

The present report deals with the occurrence of a microcytic hypochromic anemia in 9 of 13 members of a family of Sicilian descent over a span of three generations. The study was initiated when one of the members of the second generation was found to have a hypochromic anemia which did not respond to iron.

### CASE REPORT

Patient L. O., a 37 year old unmarried woman of Sicilian descent, was first seen by us on September 18, 1945, complaining of feeling faint. She had first noted this two years previously and was told by a physician that she had anemia. Therapy with iron, liver extract injections and oral liver-iron preparations were without symptomatic or hematologic benefit. She later experienced fatigue and a sensation of twitching in the left leg and hand, although no muscle contractions were ever visible. She had been studied at another hospital on two occasions but was not aware that any diagnosis was made. History of anemia in other members of her family could not be elicited.

On physical examination, the positive findings consisted of slight pallor of the mucous membranes, a metallic first heart sound at the mitral area, bilateral positive Hoffman reflex, and a nystagmus on right lateral gaze with fast component to the right. The liver and spleen were not palpable.

She had been examined by a neurologist who was unable to explain the symptoms and signs and who considered that they might be secondary to the anemia. They have never been adequately explained.

**Laboratory data.** Erythrocytes 5,570,000 per cu. mm., hemoglobin 10.4 Gm. per 100 cc., leukocytes 5,300, hematocrit 36, mean corpuscular volume 64.9 cu. microns, mean corpuscular hemoglobin 18.7 micromicrograms, mean corpuscular hemoglobin concentration 28.9 per cent. Differential blood count: 58.5 per cent neutrophils, 3.5 per cent eosinophils, 0.5 per cent basophils, 31.0 per cent lymphocytes, 6.5 per cent monocytes. No nucleated red cells were seen. The erythrocytes showed marked anisocytosis and poikilocytosis, and were microcytic hypochromic. Numerous target cells and a few oval shaped cells were present (figs. 1 and 2). The platelets were slightly increased in number. Sedimentation rate was slower than normal, the corrected value being less than zero. This finding was due presumably to delay in rouleau formation resulting from the abnormal shape of the erythrocytes.

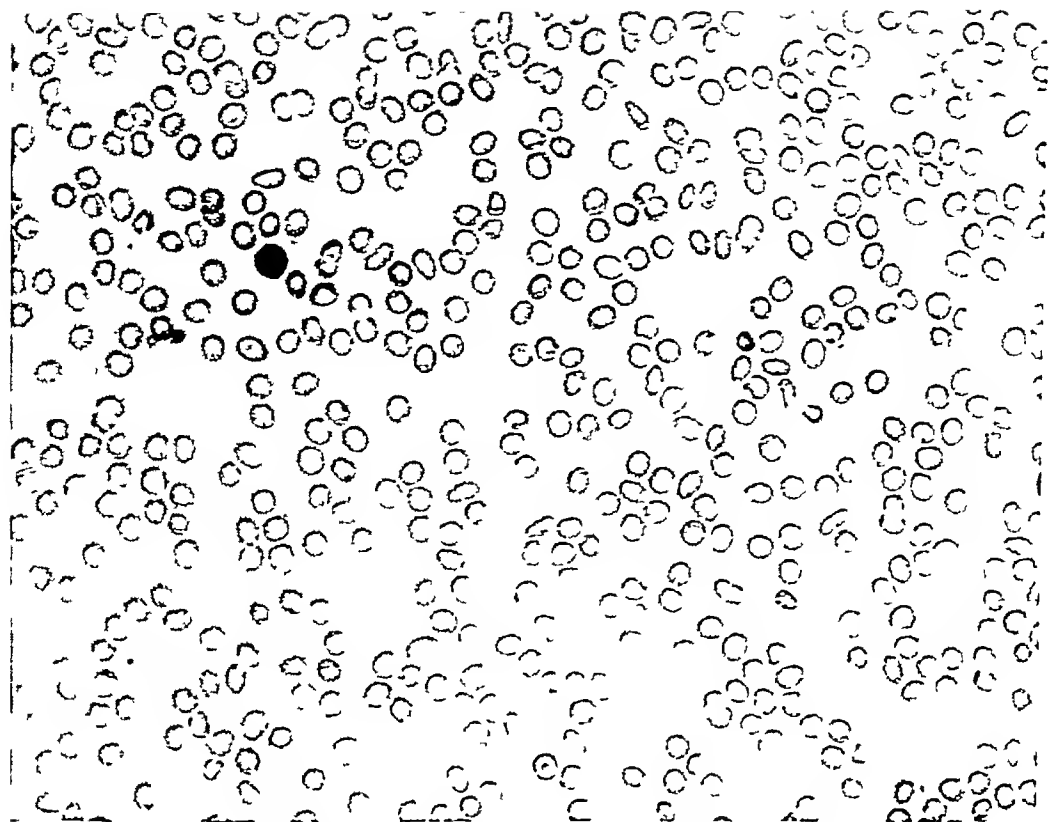


FIG. 1 Patient L. O., second generation Peripheral blood  $\times 385$

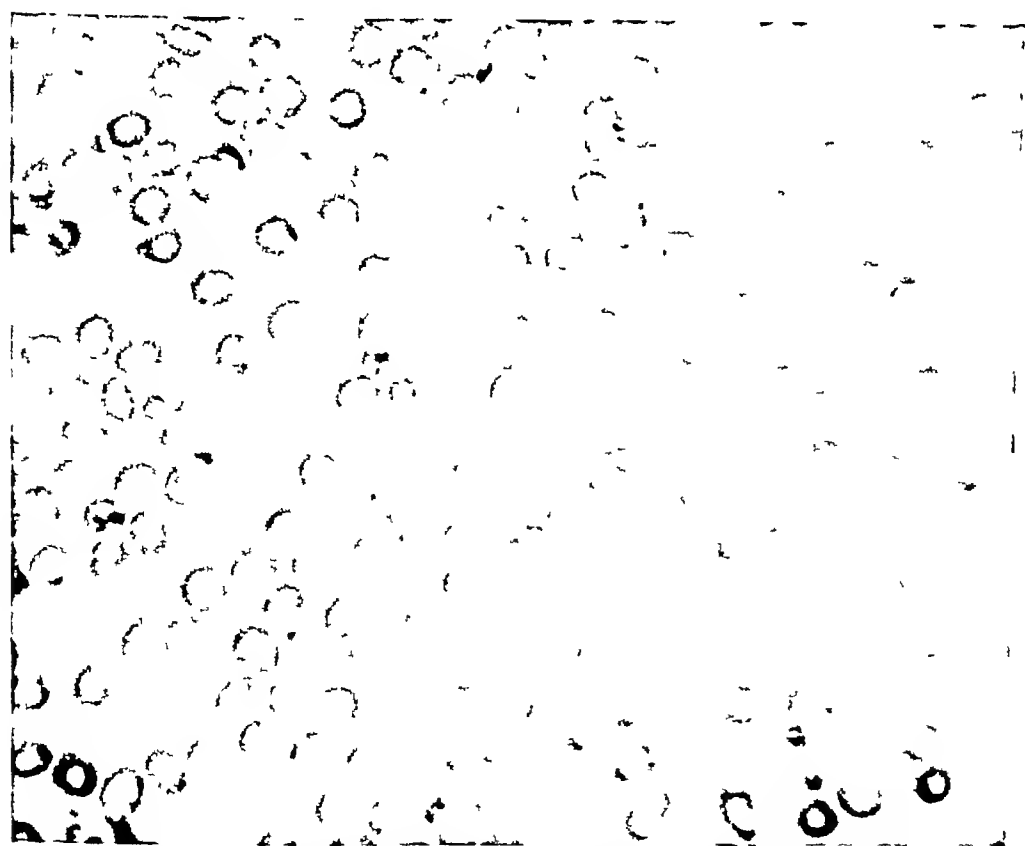
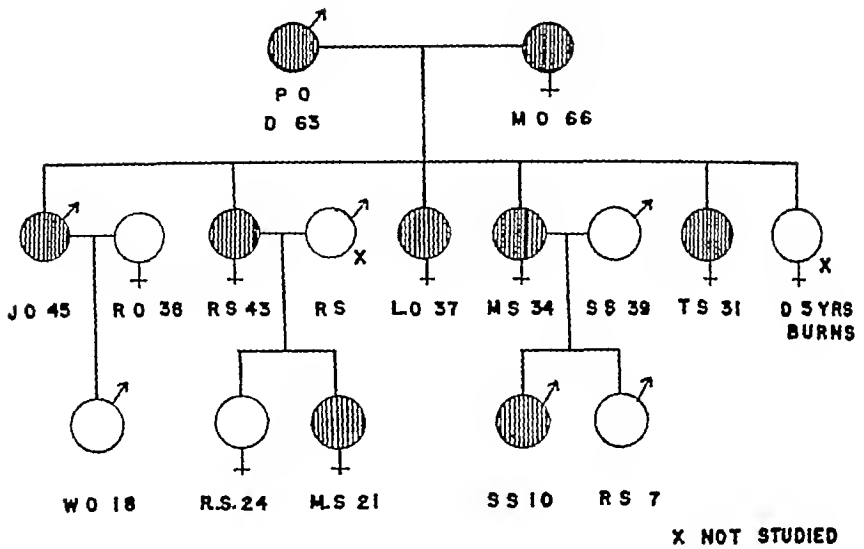


FIG. 2 Patient L. O. second generation Peripheral blood showing target cell, oval cell, polychromasia and hypochromia  $\times 640$



FAMILIAL MICROCYTIC ANEMIA

FIG 3. Occurrence of thalassemia minor in three generations of a family of Italian origin Numbers after initials indicate age of patients in years

TABLE I

Patient	Sex	Gen-eration	Age	RBC $\times 10^6$		Hgb	WBC $\times 10^3$	PCV	MCV	MCH	MCHC	Bizarre cells	Target cells	Retic	Splicen below costal margin	Ict Ind	Fragility Test % saline	
					Gm				cu mic	micro-gms	%			%	cm		Begin	Comp
P O	M	1	63	4 02	11 0	5,550				26 1		+	+					
				3 90	10 4	7,110												
M O	F	1	66	5 50	12 0	5,900	40	72 7	21 9	30 0		+	+	0		8 0	46 0	30
L O	F	2	37	5 57	10 4	5,300	36	64 9	18 7	28 9		+++	+++	0		0	38 0	20
R S	F	2	43	4 84	10 4	5,150	34 5	71 5	21 6	30 2		++	+	0 5		8 0	34 0	20
J O	M	2	45	6 33	12 7	8,500	45	71 0	20 1	28 2		++	++	2		2 0	42 0	20
T S	F	2	31	6 53	11 9	10,900	41	62 7	18 2	29 1		+	+	1		8 0	38 0	10
M S	F	2	34	5 33	11 0	10,200	38	71 4	20 8	29 2		+++	+++	0 1	0	5 0	36 0	10
M S	F	3	21	6 99	12 2	6,700	38	54 4	17 4	32 1		+++	+	0	0 5	0	42 0	22
R S	F	3	24	4 90	13 4	7,950	42 5	86 7	27 1	31 5		0	0	0 5		0	44 0	30
R S	M	3	7	5 03	13 1	10,200	41	81 5	26 0	31 2		0	0	0 3		6 0	44 0	32
S S	M	3	10	6 42	11 5	7,300	40	62 3	17 8	28 7		+	+++	0 2	0	4 0	44 0	22
W O	M	3	18	5 37	15 9	7,500	47 5	88 6	29 7	33 5		0	0	0		0	46 0	14
R O	F	S-2*	38	4 68	13 4	6,500	44	94 0	28 7	30 4		0	0	0		0	44 0	31
S S	M	S-2*	39	5 36	15 6	6,500	46	86 8	29 1	31 7		0	0	0		0		

\* S-2 = spouse of member of second generation

† Platelets estimated from smear, increased in number

‡ Platelets estimated from smear, normal in number

She was seen again one month later with the same complaints and laboratory findings There had been no response to iron therapy Saline fragility test showed beginning hemolysis at 0 38 per cent an

complete hemolysis at 0.20 per cent. X-rays of the skull, humerus and hands showed a slight degree of demineralization without specific changes.

It was felt that much of the patient's symptomatology was on a nonorganic basis, especially since she was inclined to place the blame for her symptoms on her work (machine operator), and since it seemed likely that the anemia had antedated the onset of her symptoms.

A tentative diagnosis of *familial microcytic anemia* was made and it was arranged to study other members of her family.

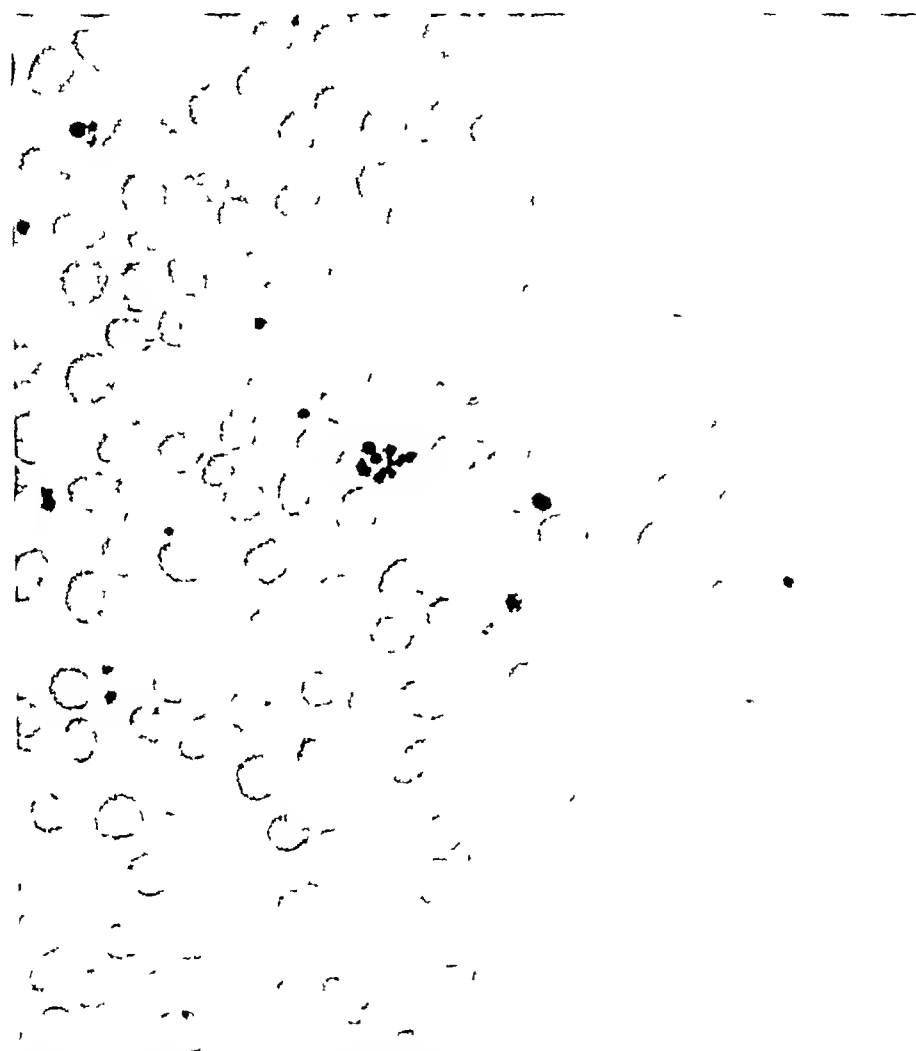


FIG. 4. Patient S. S., third generation. Peripheral blood showing hypochromia, target cells, oval cells, and poikilocytes.  $\times 640$ .

Table 1 and figure 3 show the results of findings in the other living members of the family, including the parents of the patient, her brothers and sisters, and the third generation of her nieces and nephews, in addition to two of three spouses of members of the second generation. The patient's father, P. O., had died at age 63 years, two years before we saw the patient, L. O., but he had been admitted to an outside hospital in 1942 where blood studies had been performed and where a diagnosis of duodenal ulcer and nonfunctioning gall bladder had been made. His laboratory findings were made available to us and are recorded in table 1.

A sister of the patient, L. O., died at age 5 years of burns. She was never investigated for anemia and was thought to have been entirely well. All the other members of the family, including the spouses, were in good health.

It will be noted (table 1, figure 3) that the parents of the patient, all 5 living members of the second generation and 2 of 5 members of the third generation apparently had identical blood findings. The number of target cells and bizarre forms varied somewhat but the essential features were identical. A photomicrograph of the blood of S S, a 10 year old nephew of the patient (L O) is shown in figure 4 and a photomicrograph of the blood of M S, aged 21, a niece of the patient is shown in figure 5.

The 9 affected members of this family were individuals with apparent good health, capable of doing a full day's work without unusual fatigue. There were no signs or symptoms which would have led to a diagnosis of a blood dyscrasia except the presence of palpable spleens in 5 of the 9 affected individuals.

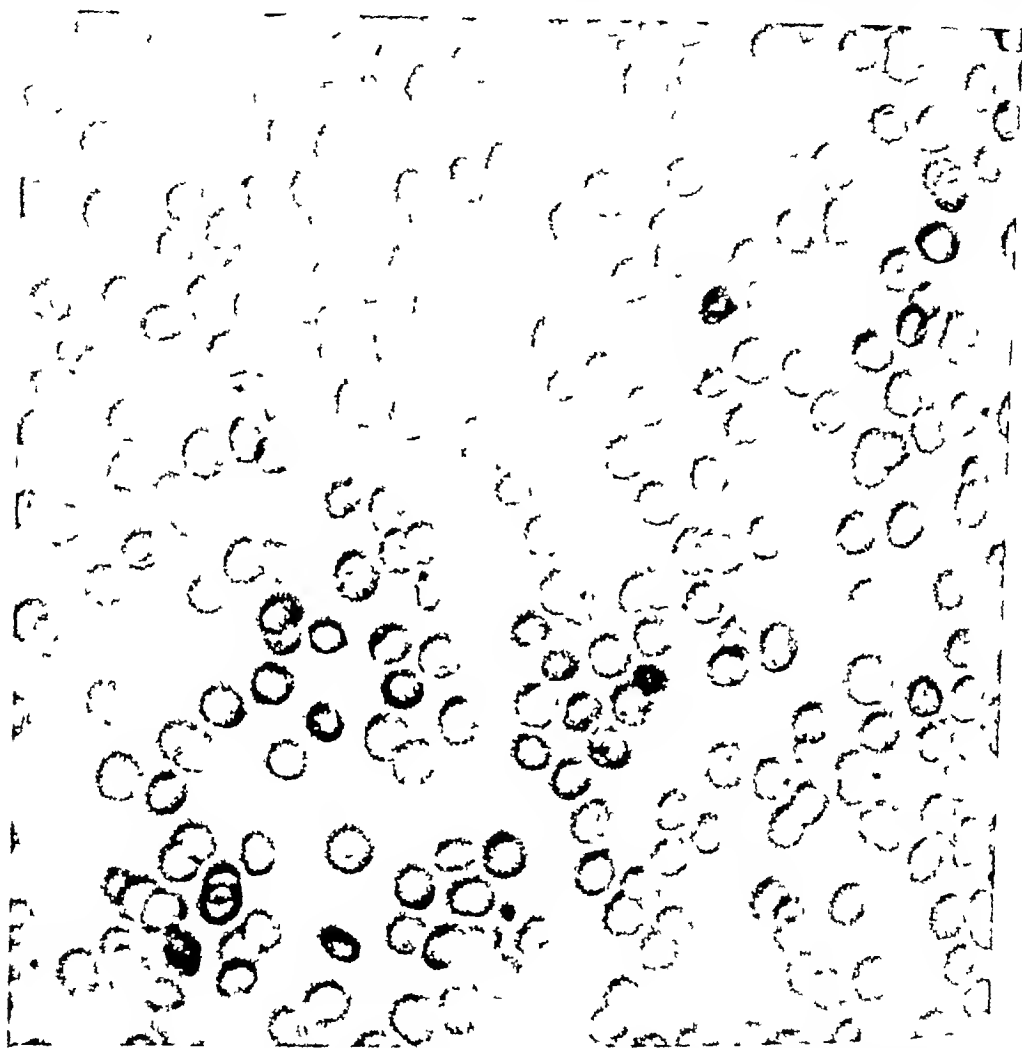


FIG. 5. M S, third generation. Peripheral blood showing target cells, oval cells and poikilocytes  $\times 640$ .

Thus, such cases are usually discovered coincidental to examination for other reasons or during a special study of a group of patients. The individuals are of Italian or Sicilian descent. The spleen is palpably enlarged in some cases but the enlargement is not great as compared with others of the blood dyscrasias, and the finding is not constant. Palpable enlargement of the liver did not occur in this series.

The erythrocyte count is frequently elevated above normal and in this series was as high as 7,000,000 per cu. mm. The hemoglobin content and hematocrit values are low, of course, so that the mean corpuscular volume and mean corpuscular hemoglobin values are considerably less than normal. The mean corpuscular hemoglobin concentration is not reduced as much, a further indication of the microcytosis. Examination of the blood films confirm these data. Hypochromia is obvious in addition to which there are variable numbers of target cells, and cells of bizarre shape. Some of the smaller erythrocytes appear to be well-filled with hemoglobin.

The saline fragility test was abnormal in all 9 of the affected individuals in this series. In some, the

molysis began at about normal concentrations, 0.42 to 0.46 per cent saline, indicating that a part of the population of erythrocytes had normal osmotic properties and were, presumably, of normal shape. In some, hemolysis began at much lower levels, indicating that all of the erythrocytes were flatter than normal. Complete hemolysis occurred at lower than normal concentrations in all cases, ranging from 0.20 to 0.24 per cent with the exception of the mother who had complete hemolysis at 0.30 per cent, only slightly below normal.

In this series there was no increase in reticulocytes, stippled erythrocytes or nucleated red cells. The leukocyte and differential blood counts were normal in all of the affected individuals. In some cases, the number of platelets appeared to be slightly increased as estimated from the blood films. Platelet counts were not made. X-rays of bones showed no specific lesions in the one case (L. O.) on whom they were made. The anemia is completely resistant to iron therapy.

### DISCUSSION

This asymptomatic, microcytic hypochromic anemia is not in itself of great importance except as the condition may fail to be diagnosed or diagnosed incorrectly. Of significance, however, is its relation to thalassemia major, a more severe disease, usually fatal during childhood. In this series, two Italians with the mild form of the disease, thalassemia minor, produced 6 children, 5 of whom are known to have had an identical mild form of the condition. It would appear at first hand, therefore, that the trait, dominant in both the parents, was simply inherited by the children. That such is not the case, however, has been demonstrated by the work of others and is further confirmed by the occurrence of the condition in the third generation of this series, in which only 2 of 5 were affected.

From the literature, and from this series, it seems probable that the severe form of the anemia, thalassemia major, results from homozygosity of an inherited factor while the milder form, thalassemia minor, results from heterozygosity of the same factor. According to this idea, both parents were heterozygous, since they had the mild form of the disease. They would be expected to have children who were homozygous (thalassemia major), heterozygous (thalassemia minor) and completely free of the trait in a ratio of 1:2:1. That all 5 of the second generation studied had thalassemia minor, signifying heterozygosity, was therefore apparently due to chance occurrence in a small sample. The statistical probability of this occurrence being due to chance is 1 in 14 (P value about 0.07, borderline significance).

The third generation in this series, however, fits the idea very well. In this case, if one parent were heterozygous and the other free of the trait, half the offspring would be expected to be heterozygous (thalassemia minor) while half would be expected to be unaffected. The occurrence of thalassemia minor in 2 of 5 members of the third generation agrees with this concept.

The mechanism of production of the abnormal erythrocytes is not understood. Valentine and Neel<sup>12</sup> were able to produce target cells experimentally, both in vitro and in vivo, by increasing the tonicity of the solution in which the cells were suspended. Whether this represents the natural mechanism of production of target cells has not been established.

### SUMMARY

1. Three generations of a family of Italian descent were studied. Nine of 13 members were found to have thalassemia minor.

2. Genetic studies indicate that this mild, microcytic hypochromic anemia characterized by the presence of target and elliptical cells and other bizarre forms and by increased resistance to hypotonic saline, results from heterozygosity of an inherited factor, which, when homozygous, produces thalassemia major or Cooley's Mediterranean anemia

3. If individuals heterozygous for this factor (thalassemia minor) marry other heterozygous individuals, one quarter of the offspring can be expected to be homozygous (thalassemia major), one half heterozygous (thalassemia minor) and one quarter free of the trait

4. The presence of thalassemia minor apparently did not interfere with the general health of affected members of this family and did not appear to shorten life expectancy. The importance of the condition lies in its relation to thalassemia major

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## PROPHYLAXIS OF HOOKWORM ANEMIA-DEFICIENCY DISEASE

By W O CRUZ, M D , AND R PIMENTA DE MELLO, M D

IN VIEW of present knowledge of hookworm anemia, it has become evident that, qualitatively and in conjunction with helminthic infestation, deficient nutrition is of importance in the genesis of this disease. The possibility of curing the anemia, even though the intestinal parasitism remains, has provided us with the opportunity of observing which symptoms and clinical signs result from a hemoglobin deficiency and which are caused directly by the presence of the helminths. Contrary to what might be expected, with the exception of intestinal hemorrhages and eosinophilia, all other pathologic changes disappeared as the blood became normal. So great is the importance of these symptoms and signs that yield with the treatment by iron, and so insignificant are those that remain, that we should, in this case, consider the anemia not as a syndrome connected with the signs, but as the disease itself. The specificity of the treatment of anemia by iron and the astonishing nature of the cure are the usual characteristics of conditions of deficiency.

Up to the present time, prophylaxis of hookworm anemia has been considered as the prophylaxis of a disease which is strictly parasitic in origin. The methods are difficult and costly, amounting almost to radical changes in the firmly established habits of a population (use of shoes) or sanitary engineering measures amounting almost to sudden civilization of backward zones (construction of privies, etc.). These classic methods of prophylaxis, consisting in avoiding the infestation of man by *Ancylostoma*, have been of no practical effect with respect to the incidence of the anemia.

If we consider the prophylaxis from the point of view of the second agent in the etiologic complex of hookworm anemia, i.e., qualitative nutritional deficiency, a different plan of prophylactic campaign can be outlined. The application of iron in prophylaxis is not sufficient to eliminate completely the disease from a community, and in addition, it requires periodic application. On the other hand, this method is one of the easiest to apply, when it is duly supported by the proper public health laws.

Following these principles, Cruz and de Mello<sup>1</sup> attempted to create the bases for a prophylaxis of hookworm anemia considered as a deficiency disease (similar to the prophylaxis of endemic goiter). This consisted in adding an iron salt, hematologically active, to the foods habitually eaten by the lower social classes. The difficulties encountered were considerable, as compared with the prophylaxis of endemic goiter. In the latter, 0.005 Gm. of potassium iodide are sufficient, whereas in hookworm anemia we had to use a much higher dose of usable iron salt. Various trials were made, not only for choice of food, but also of the iron salt with highest therapeutic value and stability. The authors concluded that the mixtures of ferrous sulfate with manioc flour and of ammoniacal ferric citrate with bean gravy were

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sufficient not only to cure, but later to prevent the fall of blood values during long periods of time, in individuals who were heavily infested. Various cases were described,<sup>1</sup> a summary of which may be seen in table 1.

With the various experimental mixtures used, it was attempted to fulfill the following requirements: taste, stability, appearance, hematologic efficiency, and low cost. Only two were found to be satisfactory: ferrous sulfate mixed with manioc flour and ferric ammonium citrate, brown, added to bean gravy. The salts

TABLE 1

Name	Age	Weight	No of worms eliminated	No of worms per kilo body weight	Intensity of infestation*	Hemoglobin level maintained (Gm/100 ml blood)	Days maintained	Iron salt used	Daily dose	Equivalent in metallic iron	Food to which added
	Yrs	kilos							Gm	Gm	
Pedro	8	23	500	22	VI	10.00	40	Ferrous sulfate	0.50	0.185	Manioc flour
Pedro	8	23	500	22	VI	9.50	85		0.25	0.092	
Argentina	20	45	717	16	VI	10.25	82		0.50	0.185	
Argentina	20	45	717	16	VI	10.25	91		0.25	0.092	
Carlos	45	50	758	15	VI	9.00	80		0.50	0.185	
Edno	13	26	350	13	V	11.00	82		0.50	0.185	
Edno	13	26	350	13	V	11.50	91		0.10	0.037	
Jose V	16	38	499	13	V	11.25	112		0.50	0.185	
Jose V	16	38	499	13	V	11.00	45		0.25	0.092	
J. Magalhães	9	28	300	11	IV	10.25	90	Ammoniacal ferric citrate	1.00	0.210	Bean gravy
Valdir	12	30	280	9	IV	9.50	87	Ferrous sulfate	0.50	0.185	Manioc flour
Mario	18	46	230	5	IV	10.00	65		0.50	0.185	
Mario	18	46	230	5	IV	10.00	128		0.25	0.092	
Delvaire	9	26	130	5	IV	10.50	87		0.50	0.185	
Maria	19	45	180	4	III	11.00	80		0.50	0.185	
Maria	19	45	180	4	III	11.75	90		0.10	0.037	

\* See table 3

listed in table 2 were also tried, but did not fulfill the requirements and their use was not continued.

With regard to the infestation index listed in table 1, we should keep in mind that, according to present knowledge, the contribution of the helminths to the formation of anemia appears to be exclusively through their blood-sucking activities. The hemorrhages caused by this action have a distinctive significance in the physiology of the blood. The organism reacts in various ways according to the hematic constituents lost in a hemorrhage. It seems to possess an unlimited quantity of protein for reconstitution of the red blood cell stroma, of globin, and of amino radicals present in the chemical structure of heme. This is not the case with

relation to the basic metal for the respiratory function. This metal is a vital raw material for reconstitution of respiratory pigment, and the organism is entirely dependent on the reserves supplied to it by nutrition to maintain a normal hemo-

TABLE 2

<i>Iron Compound</i>	<i>Food to which added</i>	<i>Color of mixture</i>	<i>Taste</i>	<i>Hematologic activity in therapeutic dose</i>
Iron carbonate	Kitchen salt	brown	o	—
" "	Flour	brown	o	—
" "	Sugar	brown	o	—
Iron glycerophosphate	Kitchen salt	yellowish	+	+
" "	Kitchen salt	o	+	+
" "	Sugar	yellowish	+	+
Iron proto-oxalate	Kitchen salt	yellowish	o	+
" "	Flour	yellowish	o	+
" "	Sugar	yellowish	+	+
Iron pyrophosphate	Kitchen salt	white	o	+
" "	Flour	o	+	+
" "	Sugar	o	+	+
Ammoniacal iron sulfate	Kitchen salt	dark yellow	+++	++
" " "	Flour	o	+++	++
" " "	Sugar	darkish	+++	++
" " "	Sugar	darkish	++	++
Iron phosphate	Kitchen salt	dark green	+	++
" "	Flour	grey	+	++
" "	Sugar	greenish grey	+	++
Iron albuminate	Kitchen salt	brown	o	—
" "	Flour	brown	o	—
" "	Sugar	brown	o	—
Ammoniacal ferric citrate	Kitchen salt	yellow	+++	++
" " "	Flour	o	+++	++
Tartrate of iron and potassium	Kitchen salt	light brown	++	—
" " " " "	Kitchen salt	light brown	++	—
" " " " "	Flour	brown	o	—
" " " " "	Sugar	dark brown	o	—
Iron benzoate	Kitchen salt	dark brown	+++	—
" "	Flour	dark brown	+++	—
" "	Sugar	dark brown	+++	—
Iron lactate	Kitchen salt	greenish	+++	+
" "	Flour	o	++	+
" "	Sugar	brown	++	+
Ferrous sulfate	Kitchen salt	yellow	++	++++
" "	Flour	o	o	++++
" "	Sugar	yellow	++	—

globin metabolism. Accordingly, when the helminths withdraw blood from the body, they withdraw essentially the iron metal. Therefore, each helminth represents a unit of consumption in the iron balance in the body. This unit will increase in importance in proportion to the decrease of iron in the circulation of the host. It is known that in mammals the total amount of blood is approximately 10 per

cent of the body weight. Hence, the damage caused by a worm will be less important in an adult of 60 kilos than in a child of 20 kilos. This means that the intensity of infestation can be expressed only by a relationship between the number of worms living on the intestine and the mass of circulating blood, or roughly the body weight of the host. Based on these data, we suggest that the intensity of infestation from *Ancylostoma* be figured according to table 3.

In order to determine approximately the number of helminths per kilo of body weight, based on egg counts, the following formula is used  $\frac{N}{18P}$ , in which N represents the number of eggs per gram of feces and P the weight of the individual expressed in kilograms. Usually the infestation occurs with an equal number of male and female helminths, and as the females of the *Necator* are responsible for eliminating 36 eggs per gram of stools, we should divide the egg count by half of 36, which explains the factor 18 in the denominator of the formula. Therefore, for

TABLE 3

Intensity of infestation—Groups	Helminths per kilo of body weight
I	0
II	0-0.9
III	1-4.9
IV	5-9.9
V	10-14.9
VI	over 15

example in a child, 31 kilo body weight, with 5,000 eggs per gram of feces we have  $\frac{5000}{560} = 8.9$  helminths per kilo of body weight, a case belonging to group IV of our classification.

Following these studies on the administration of iron in the prophylaxis of hookworm anemia considered as a deficiency disease, it would doubtless be very important to determine the minimum dose of salt to be used, in order to maintain the blood values at a normal level. For this purpose, we submitted a patient, with a high index of infestation, to several doses of ferrous sulfate added to the food.

### CASE REPORT

C. G., 22 years old, railroad worker, white, Brazilian, resident of Magé. Weight, 45 kilos. Admitted to the hospital on January 11, 1946. Discharged April 4, 1947.

Patient complains of extreme weakness, is easily tired, has dyspnea and palpitation after making the slightest physical effort. Can not say for certain when illness commenced, the symptoms appeared and progressed in unnoticeable manner. Says he had no venereal or rheumatic past. Although living in a malaria zone, informs never had malaria. Drinks alcohol in moderation.

**General examination.** Asthenic, badly nourished individual. Skin yellowed, visible mucosae highly discolored, almost white. Lesions of chronic scabies spread over trunk, abdomen, base of thighs, and hands. In the malar region on both sides and as far as the edges of the nose, symmetrical, irregular zones of dark coloring and a little shiny can be noted. On malleoli slight edema, less than one month old. No decrease or changes in appetite. Teeth are in poor condition. Tongue is white, broadened, and marked. Teeth can be seen on tip.

*Digestive system* Epigastric region is sensitive to touch, but does not present spontaneous pain. No constipation, in last two months, attacks of diarrhea have been frequent. Liver and spleen not increased in volume.

*Circulatory system* Pulse light, soft, and rhythmic, with 84 pulse beats per minute. Blood pressure is 110/75. Lack of thrill in neck vessels. Ictus weak, located in fifth intercostal space, one centimeter inside the hemi-clavicular line. Systolic murmur (++) soft, audible at point and at base. Not spread by any focus. Diminishes in intensity at beginning of inspiration, and, on the other hand, increases when the individual lies down or when the auscultation point is pressed with stethoscope. In the pre-systole, the auricular sound is heard in the mesocardiac region. A<sub>2</sub> and P<sub>2</sub> are equal and normal.

*Respiratory system and other systems, normal*

*Sequence in hospital* The stay of the patient in the hospital was not apyrexial due to two factors not connected with the *Ancylostomiasis* (1) a secondary infection in some lesions of the scabies mentioned, (2) a dental abscess, both occurring when health conditions were very poor. With the use of iron, the symptoms and signs caused by the anemia diminished immediately. At the end of the first week, the

TABLE 4 — *Hematologic Tests*

	Date													
	1946										1947			
	1 18	1 25	2 4	2 15	2 25	4 19	5 21	8 12	9 26	11 19	1 3	2 6	4 12	
	Days of observation													
	0	7	16	27	37	91	123	204	248	301	345	378	444	
Red blood cells (10 <sup>6</sup> /ml)	1 2	1 9	2 6	3 7	3 7	4 9	5 1	4 5	4 8	4 6	4 3	3 8	5 0	
Hemoglobin (Gm/100 ml blood)	1 5	4 4	6 2	8 8	8 2	11 2	12 0	9 6	11 2	9 0	10 2	8 6	11 6	
Hematocrit (%)	7	14	21	29	30	35	38	34	36	33	32	29	38	
Mean corpuscular volume (cubic micra)	58	73	80	78	80	71	75	76	75	72	75	76	76	
Mean corpuscular hemoglobin (micro-micrograms)	12	23	24	24	22	23	23	21	23	20	24	23	23	
Mean corpuscular hemoglobin concentration (%)	21	31	29	30	27	32	32	28	31	27	32	30	31	

malleolar edema no longer existed. Urine examinations, made immediately after the patient was admitted to the hospital, and subsequent examinations, showed nothing to indicate that renal function was affected. Appetite was always good. The attacks of diarrhea disappeared. Forty days following beginning of treatment, the patient had gained 6 kilos weight. Color of skin and of mucosas practically normal, for our environment, at end of February, that is, 45 days after admittance. Tongue had regained tonus. Physical resistance permitted the practice of active exercise without reappearance of dyspnea and palpitations. Heart beat remained about 70 per minute. Blood pressure not changed, systole continuing between 105 and 115 and diastole at 75 mm Hg. Beginning the middle of March, systolic murmur no longer heard, only first sound found to be extended at point. Auricular sound heard only when the heart, because of the requirements of physical effort, became hyperactive. No opportunity to make radiologic study of this case.

We accompanied clinical course of the anemia with frequent electrocardiograms. We will analyze only two, spaced about three months apart. The others are transitional between these two, or repeat the second, which represents, so to speak, the final modification observed.

In figure 1 (January 16, 1946) and figure 2 (April 9, 1946), the second (fig 2) shows the following modifications when compared with the first.

- 1 Slight rotation of electric axis of the QRS to the left
- 2 Increased voltage on wave-length T in D<sub>1</sub> and in precordial positions left of the ictus,
- 3 Positivity of wave-length T in V<sub>3</sub>

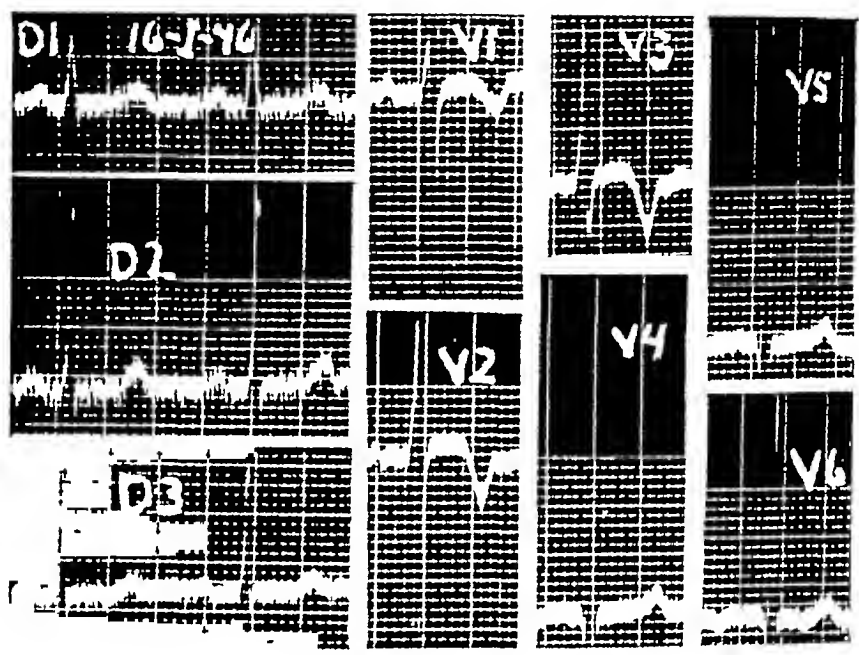


FIG. 1

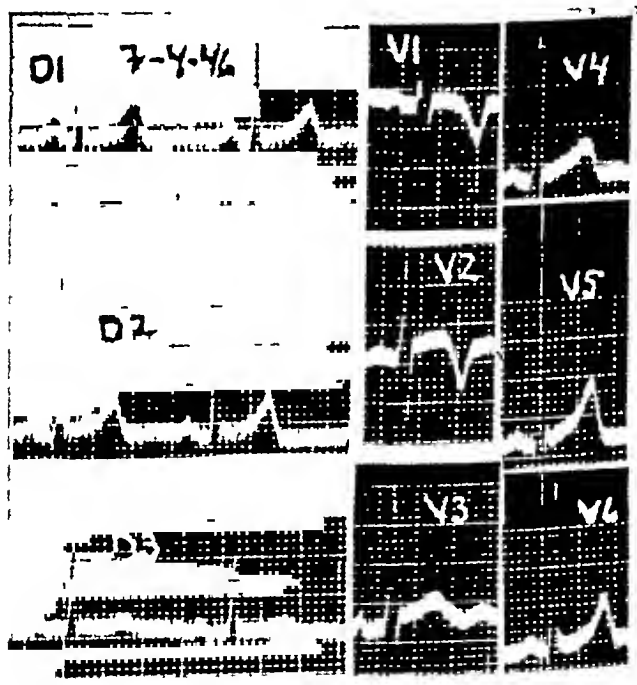


FIG. 2

The electrocardiographic changes may partly be due to changes in the position of the heart. Increase of wave-length T in precordial positions left of the ictus in

Dr is probably due to changes of the process of repolarization of ventricular myocardium, caused by better nutritive conditions of muscular fibers

We started the therapy with iron, administering ferrous sulfate, 10 gram daily mixed with manioc flour, a food widely used in certain regions of Brazil. The blood values increased rapidly from 20 grams to 70 grams of hemoglobin per 100 cc of blood. We decreased the dose to 0.5 Gm daily, always added to the same food. At the end of two months, the hemoglobin value was practically normal (110 grams per 100 cc of blood). We then tried to determine the minimum dose necessary to maintain a relatively normal hemoglobin level. The administration of 0.1 Gm daily was insufficient to maintain this level, and hemoglobin decreased from 110 Gm to 80 Gm at the end of 110 days. Experiments with 0.2 Gm of ferrous sulfate, however, proved to be a sufficient dose to avoid the decrease, and enough

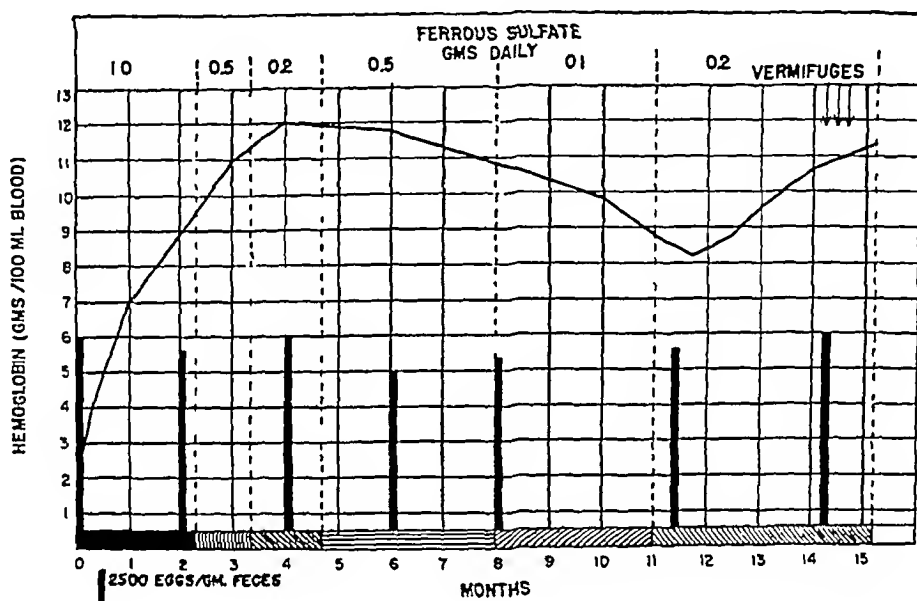


FIG 3

to keep the blood levels normal for 100 days. A graphic presentation of this observation is given in figure 3.

Attention should be called to the extreme clinical changes which occurred in this patient after iron was administered. When he was hospitalized, the patient was entirely apathetic, without strength to move, remaining in bed for a considerable portion of the day. As the blood values became normal, his functional activity was restored. In the final period of treatment, he remained semi-hospitalized, and worked in our laboratory. In carrying out his work, he walked daily about two kilometers from the hospital to the place of work. He became quite active, as can be seen by the fact that several times a day he went to the animal house, about 200 meters away, going up a steep incline and climbing three flights of stairs on returning to the laboratory. He no longer felt the symptoms of which he complained when he was hospitalized. He became, from all points of view, a perfectly normal individual.

Eggs of *Necator* were counted periodically, for control of the biologic activity of the helminth and the persistence of the degree of infestation

At the end of the trial period, five vermifuges (carbon tetrachloride 1.8 ml + *Chenopodium* oil 0.6 ml) were administered at weekly intervals. Helminths to the number of 1051 were eliminated, representing one of the most heavily infested cases we have observed (infestation index = 24 helminths per kilo of body weight). The fact should be kept in mind that the number of helminths eliminated represents a minimum, since it is easy to understand that not only do some escape at time of counting, but also others disappeared by natural death during the period of hospitalization.

### SUMMARY

1 In individuals severely infested with *Ancylostoma* or *Necator*, it is possible to maintain the normality of blood value by the administration of a sufficient dose of an iron salt.

2 The minimum dose necessary to maintain normality of the blood in an individual weighing 45 kilograms, with 1051 helminths, was 0.2 Gm daily of ferrous sulfate, administered in mixture with manioc flour.

3 The patient observed became clinically normal two weeks after the beginning of blood regeneration up to the end of the trial period one year later. In this period, with the various doses of iron tried, hemoglobin varied from 8.0 to 11.0 per 100 ml of blood.

### ACKNOWLEDGMENT

We owe thanks to the kindness of our colleague, Dr Genard Nobrega, for the case report and electrocardiographic study of the patient.

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# A STUDY OF SICKLING OF YOUNG ERYTHROCYTES IN SICKLE CELL ANEMIA

By JANET WATSON, M D

THE COMPARATIVE scarcity of sickled reticulocytes and normoblasts in patients with active sickle cell disease has been sufficiently striking to arouse comment by several students of that disease <sup>1 1a 2 3</sup> This observation led Murphy and Shapiro<sup>3</sup> to postulate that the occurrence of hemolytic crises might be due in part to the increasing tendency of the red cells to sickle on aging, in which case the reticulocytosis of a crisis might of itself be beneficial The fact that their patient showed a higher percentage of sickle cells in the plain smear before crises than afterwards seemed to lend further support to this theory In reviewing many blood smears of sickle cell patients, I was able to find only two normoblasts and one reticulocyte in the sickled form A quantitative study of the sickling of reticulocytes was therefore undertaken

## MATERIAL

Three patients with active sickle cell anemia from the Hematology Clinic of Kings County Hospital were chosen for study because they consistently showed a high percentage of reticulocytes and sickle cells in their blood The Wintrobe oxalate mixture was used as an anticoagulant for the venous blood obtained

## METHODS AND RESULTS

### 1. *Sealed Preparation*

(a) *Blood* Oxalated blood was mixed with equal parts of 0.5 per cent brilliant cresyl blue in 0.85 per cent NaCl solution One drop of the mixture was used to make the standard slide-cover slip preparation This was sealed with paraffin, incubated at 37°C, and examined for sickling at frequent intervals In all cases sickling was complete within two to four hours This included the reticulocytes as well as the occasional normoblasts present It was the impression that most of the reticulocytes sickled as soon as the mature red cells, with the exception of some of the most immature reticulocytes which were packed full of reticulum The normoblasts appeared to be the last to sickle The progressive sickling of the reticulocytes could not be counted accurately by this method, however, because of the irregularity of sickling in different parts of the same preparation This irregularity has been mentioned by others, and is probably largely due to uneven distribution of the leukocytes, which have an accelerating effect on the sickling of the red blood cells,<sup>4 5</sup> presumably through lowering oxygen tension by metabolism Platelet distribution may also be a factor <sup>6</sup>

(b) *Bone Marrow* One cc of sternal marrow was obtained from one patient (W. B.) The buffy layer was used in a sealed preparation, made as described above, in order to study the sickling of normoblasts Cresyl blue stains the cytoplasm of the basophilic and polychromatophilic normoblasts, but not that of the orthochromatic normoblasts unless it happens to be reticulated The reticular network is easily distinguished from the diffuse blue staining of the basophilic normoblasts It was found that none of the basophilic and polychromatophilic normoblasts were sickled after 24 hours incubation at 37°C, whereas most of the orthochromatic normoblasts were in the sickled state Two of the latter type containing Howell-Jolly bodies, however, failed to sickle Since the orthochromatic normoblasts have a full quota of hemoglobin in their cytoplasm, their ability to sickle is not surprising Further evidence for the primary role of hemoglobin in sickling has been presented recently by Ponder,<sup>7</sup> who showed that when meniscocytes were made into ghosts by lysis of their hemoglobin, they lost their ability to sickle



2. *Gas Chamber Method*

The apparatus used was essentially that described by Hahn and Gillespie<sup>8</sup> except that the chamber was made of paraffin instead of glass. A water sealed outlet was found necessary. Both carbon dioxide and nitrogen were used for sickling. The same saline cresyl blue blood mixture was used. Since the red cells in the hanging drop can be studied directly with the oil immersion lens, this method has the advantage that the active dynamic process of sickling can be watched easily. Here again most of the reticulocytes seemed to sickle as fast as the other red cells, but the great rapidity of the sickling—about two minutes for complete sickling—made impossible the quantitative timing of the transformation of the two types of cells. Rarely would the rearrangement of the hemoglobin in the process of sickling result in the reticulum being lost from view. Occasionally the formation of the Sherman 'holly wreath' forms of sickle cells<sup>4</sup> made this method, as well as that of the sealed preparation, unsatisfactory. Because of these disadvantages a chamber method which would permit slower sickling and the periodic removal of cells for counting purposes was devised as follows.

3. *Gas Test Tube Method*

A test tube, 15 x 40 mm, with a capacity of 5 cc. was set up with a gas inflow and outflow via 24 gage needles through a rubber stopper. The outflow was equipped with a water seal. Carbon dioxide was used

TABLE 1—*Progressive Sickling of Reticulocytes upon Aeration with Carbon Dioxide*

Patient	Time in CO <sub>2</sub>	Reticulocytes (per 100 RBC)	Sickle Cells (per 100 RBC)	Sickled Reticulocytes (per 100 RBC)	Sickled Reticulocytes (per 100 retics)
	<i>min</i>				
L J	0	10 5	9 5	0 0	0 0
	2	10 0	45 0	4 5	45 0
	5	9 0	81 0	7 0	77 7
	10	9 0	92 5	8 5	94 4
W B	0	20 5	15 0	0 0	0 0
	2	19 5	28 0	4 0	20 5
	5	21 0	69 5	13 5	64 3
	10	20 0	90 0	17 5	87 5
J W	0	15 0	11 5	0 0	0 0
	2	16 5	33 5	4 5	27 3
	5	14 0	76 0	9 5	67 8
	10	14 5	93 5	13 0	89 6

for sickling. One cc. of the saline cresyl blue blood mixture was introduced into the inverted test tube. Small samples of blood were removed at appropriate intervals under oil with an oiled tuberculin syringe and a 22-gage needle through the rubber stopper. The blood was immediately injected into formalin for fixation. Reticulocyte and sickle cell counts had to be done immediately in order to avoid inaccuracy due to slow fading of the reticulum in formalin. A 2 per cent formalin solution in normal saline was found to be as good a fixative as the standard 10 per cent solution and had less of a fading effect. A drop of the red cell suspension was placed on a slide under a cover slip, and a count was made under oil immersion of the reticulocytes and sickle cells. Only 200 cells were counted because of the time factor of fading.

The data obtained are shown in table 1. The number of sickled reticulocytes found per 100 RBC was divided by the number of reticulocytes per 100 RBC in order to calculate the percentage of sickled reticulocytes in terms of total reticulo-

cytes By comparing these figures with those for the percentage of total sickled cells, it is evident that the rate of sickling of the reticulocytes is quite similar to the rate of sickling of the whole red cell population A reticulocyte in the sickled form is shown in figure 1

A possible theoretic objection to the fact that sickle cells are not reticulated in an ordinary smear is that the abnormal shape interferes with the supravital staining In answer to this objection, blood was completely sickled in the test tube chamber, after which an equal amount of saline cresyl blue, previously aerated with carbon dioxide, was introduced into the test tube under oil The blood was examined after two minutes, and the sickled reticulocytes were found to be well stained

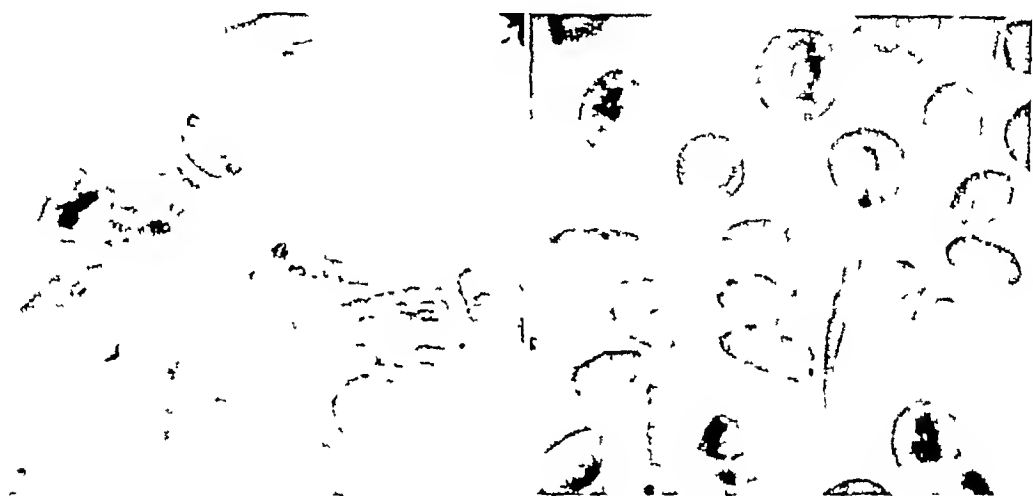


FIG 1

FIG 2

FIGURE 1 Meniscocytes sickled by carbon dioxide, stained with cresyl blue and immediately counterstained with Wright's stain Cells are shown in the process of unsickling with transition forms from the sickled shape to the biconcave disk Note the sickled reticulocyte at the left Two crescent forms have developed elongated processes at the ends The black spots with refractile contours are artefacts in photography

FIGURE 2 Peripheral blood smear stained with cresyl blue and Wright's stain Three reticulocytes and seven crescent, elliptical and oval shaped sickle cells can be seen None of the sickle cells are reticulated These sickled cells lack the bizarre shapes seen in Fig 1

In all three methods used, sickling of all erythrocytes and normoblasts was quickly reversible within a few seconds upon admission of oxygen to the system

#### DISCUSSION

Direct counting of the progressive sickling of reticulocytes on aeration with carbon dioxide (table 1) by means of a gas test tube chamber has shown that most reticulocytes sickle as well as the more mature cells However, it was noted in this and in the other methods used that the reticulocytes with the largest amount of reticulum were usually late in sickling The few normoblasts observed were also somewhat slow Although this may be due to the possibility that the most immature cells have a lower oxygen tension threshold for sickling, there is an al-

ternative explanation that the presence of a nucleus or of a large quantity of stain reticulum may mechanically interfere with the sickling process

The virtual absence of reticulated sickle cells in the ordinary smear (fig. 2) seems paradoxical at first glance. The sickle cells seen in the fixed blood smear appear different from the ones produced by *in vitro* sickling.<sup>2, 6, 9</sup> The former are crescent shaped, elliptic, or oval and do not have the long processes seen in sickled preparations. When viewed directly in a hanging drop, these forms are seen to send out one or more elongated processes at the pointed ends on aeration with carbon dioxide, nitrogen, and to revert to their original shape on aeration with oxygen without going on to the normal discoid forms. For this reason these abortive sickle cells should perhaps have a distinctive terminology. It may be that they are old cells that have lost their "elasticity" from being kept in the sickled shape for long periods of time in stagnant blood vessels. Good evidence for the role of stagnation is presented by Diggs and Bibb<sup>2</sup> who had three patients with sickle cell anemia who had irreversible sickle cells in their pleural or ascitic fluid, although they had none in their stained blood smears. It should be possible to produce these crescent forms *in vitro* by keeping cells in their sickled shape for long periods of time, but attempts so far have been unsuccessful.\* It is a well known fact that sickle cells are not seen in the blood smears of persons with the sickle cell trait. This could be predicted since the oxygen tension necessary for *in vitro* sickling (18 mm Hg)<sup>4</sup> would never occur *in vivo*.

Reticulocytes, being young cells, may have more "elasticity," so that they are not fixed in the sickled shape *in vivo*. Or it may be that the length of time for stagnation of erythrocytes to produce these abnormal forms may surpass the estimated five to six day life span of the reticulocyte.<sup>10</sup> If we accept Tomlinson's finding<sup>11</sup> that sickled cells are actually stuck in the interstices of the spleen pulp and cannot be perfused out, it would seem that the spleen could be an important dragnet of sickled erythrocytes in those patients in whom that organ had not become entirely fibrotic.

There is disagreement as to whether the number of circulating sickle cells differs from time to time in the same patient. Diggs and Bibb<sup>2</sup> and Smith<sup>12</sup> found little variation, while Sydenstricker<sup>13</sup> and Murphy and Shapiro<sup>3</sup> found that the sickle cells increased before the crisis and fell after the onset. Emmel<sup>14</sup> also noted significant variation in his patient. The high viscosity of sickled cells<sup>15</sup> and their abnormal shape tend toward their sequestration in organs, so that there could be an increasing accumulation of sickled forms without this increase necessarily being reflected in the peripheral blood. If this is so, the diverse reports among various investigators is not surprising. The increased mechanical fragility of the sickled cells<sup>2, 16</sup> must be an important factor in their final demolition. Thus, a crisis results in the destruction of the old sickled cells and in the outpouring of new young red cells. Although the reticulocytes appear to sickle as well as other cells, they are

\* A personal communication from Dr. Shu Chu Shen indicates that sterile incubation *in vitro* of erythrocytes maintained in the sickled form renders them unable to reassume the discoid form upon exposure to oxygen. The technic employed was the sterile incubation of defibrinated blood samples from either anemic patients or those showing the trait for only twenty-four hours. This incubation was carried out after preliminary equilibration with and during continuous exposure to a gas mixture composed of 90 per cent nitrogen and 10 per cent carbon dioxide.

not found in the irreversibly sickled shape. As the maturing red cell stagnates in anoxic organs, irreversible sickle forms appear, and augment the vicious cycle of stagnation, anoxemia, increasing sickling, thrombosis, and hemolysis.

### SUMMARY

1 Data have been presented to show that most reticulocytes from patients with the sickle cell trait or sickle cell anemia sickle as readily as do more mature red blood cells. The most immature reticulocytes and normoblasts tend to sickle more slowly.

2 Orthochromatic normoblasts were the only type of normoblasts which sickled, the basophilic and polychromatophilic types could not be sickled.

3 It is suggested that the sickle cell forms seen in ordinary stained smears represent old cells which have lost their "elasticity" while stagnating in the sickle shape, and are unable to revert to a biconcave disk. This would explain the fact that these forms are so rarely found to be reticulated when stained with brilliant cresyl blue.

### ACKNOWLEDGMENT

I wish to express my gratitude to Dr. William B. Castle, Dr. William Dock (Department of Medicine) for many stimulating suggestions, to Dr. John M. Pearce (Department of Pathology) for assistance in preparing this report and to Mrs. Muriel MacDowell (Department of Pathology) for the photomicrography.

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# BLOOD

## *The Journal of Hematology*

VOL III, NO 5

MAY, 1948

*This issue is the third part of the George Richards Minot Anniversary Volume*

### THE CHEMICAL SPECIFICITY OF THE INTERACTION OF DIVERSE HUMAN PLASMA PROTEINS

By EDWIN J COHN, PH D

RECOGNITION of the specific chemical functions of the various protein components of the plasma began in the eighteenth century. In his book, 'An Experimental Inquiry into the Properties of the Blood,' published in 1771, Hewson (fig 1) described the separation of fibrinogen from plasma.<sup>1</sup>

When fresh blood is received into a bason, and suffered to rest, in a few minutes it jellies, or coagulates, and soon after separates into two parts, distinguished by the names of *Crassamentum* and *Serum*.

It is well known, that the *crassamentum* consists of two parts, of which one gives it solidity, and is by some called the fibrous part of the blood, or the *gluten*, but by others with more propriety termed the *coagulable lymph*, and of another, which gives the red colour to the blood, and is called the *red globules*. These two parts can be separated by washing the *crassamentum* in water, the red particles dissolving in the water, whilst the coagulable lymph remains solid. That it is the coagulable lymph, which, by its becoming solid, gives firmness to the *crassamentum*, is proved by agitating fresh blood with a stick, so as to collect this substance on the stick, in which case the rest of the blood remains fluid\*.

\*It may be proper to mention here, that till of late the coagulable lymph has been confounded with the serum of the blood, which contains a substance that is likewise coagulable. But in these papers, by the *lymph*, is always meant that part of the blood which jellies, or becomes solid spontaneously when blood is received into a bason, which the coagulable matter that is dissolved in the serum does not, but agrees more with the white of an egg, in remaining fluid when exposed to the air, and coagulating when exposed to heat, or when mixed with ardent spirits, or some other chemical substances.

Hewson thus recognized the water-soluble constituent of the red globules, the fibrinogen of the *crassamentum* or clot, and the coagulable proteins, largely the albumins, of the serum. Serum proteins, insoluble in water at a slightly acid reaction but dissolved by salt, were recognized by Denis and Scherer in 1841.<sup>2</sup> Over a century ago, therefore, the chief protein component of the red cells and at least three protein components of plasma had been recognized.

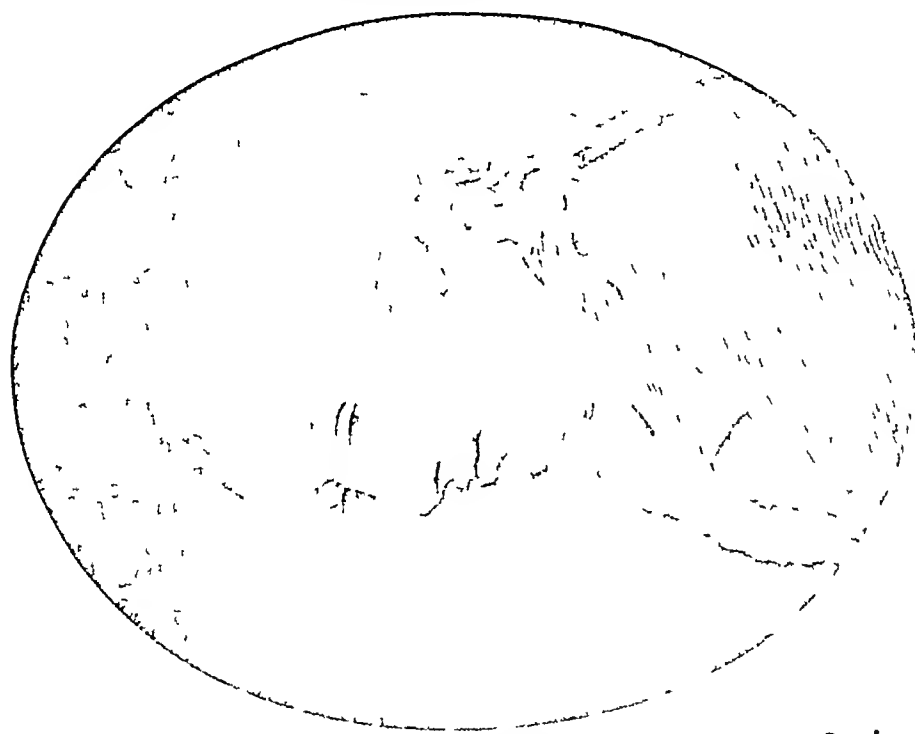
We now know that the pigment of the red globules, largely responsible for the respiratory function of the blood, is the prosthetic group of the protein, hemoglobin, and that the red blood cells contain in addition a large number of recently discovered protein components, among them carbonic anhydrase, catalase, phosphatase, choline esterase, hypertensinase and other peptidases. In this communica-

This paper is Number 63 in the series 'Studies on Plasma Proteins' from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

It is well known, that the *crassamentum* consists of two parts, of which one gives it solidity, and is by some called the fibrous part of the blood, or the *gluten*, but by others with more propriety termed the *coagulable lymph*; and of another, which gives the red colour to the blood, and is called the *red globules*. These two parts can be separated by washing the *crassamentum* in water, the red particles dissolving in the water, whilst the coagulable lymph remains solid. That it is the coagulable lymph, which, by its becoming solid, gives firmness to the *crassamentum*, is proved by agitating fresh blood with a stick, so as to collect this substance on the stick, in which case the rest of the blood remains fluid \*.

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his portrait and excerpts



WILLIAM HEWSON

(1739-1774)

An Experimental Inquiry into the Properties of the Blood

The Preface of Hewson's work

ed,

a

Fig 1

# AN EXPERIMENTAL INQUIRY

INTO THE

PROPERTIES of the BLOOD.

P R E F A C E.

THE knowledge of the human frame, the preservation of health, and the cure of diseases, are objects of too great importance to mankind, for the Author of these sheets to doubt, that any attempts to promote them, how small soever, should not meet with a candid and indulgent reception from the public. An Inquiry into the Properties of the Blood, it is presumed, will be thought, in a particular manner, interesting, since there is no part of the human body upon which more physiological reasoning is found-

ed,

a

Fig 1

tion we shall concern ourselves, however, with the plasma proteins and the specific chemical reactions upon which their physiologic functions depend

The relation of fibrinogen to the clotting of the blood was implicit in Hewson's discovery. Although Hewson found ways of blocking fibrin formation it remained for Andrew Buchanan to demonstrate, in 1845, 'that fibrin has not the least tendency to deposit itself spontaneously in the form of a coagulum that like albumen and casein, fibrin only coagulates under the influence of suitable reagents and that the blood, and most other liquids of the body which appear to coagulate spontaneously, only do so, in consequence of their containing at once fibrin and substances capable of reacting upon it, and so occasioning coagulation' <sup>3</sup> The substance which Buchanan noted, by virtue of its physiologic action, was presumably thrombin. Satisfactory studies upon thrombin as a globulin, were, however, not carried out until this century <sup>4-9</sup>

Meanwhile albumins and other globulins had been noted in terms of chemical properties, often however, without these properties being associated with specific biologic functions. The chemical studies which have continued during the last century have yielded relatively pure preparations of albumins and demonstrated that there were many kinds of globulins.

*Molecular Dimensions of Plasma Proteins* Although there is evidence of more than one albumin <sup>10-15</sup> all serum albumins which have been studied thus far, of human or animal origin, have closely similar molecular properties, isoelectric points near pH 4.9, molecular weights near 70,000 and molecular dimensions described as ellipsoids 150 Ångströms in length and 38 Ångströms in width. All plasma proteins thus far investigated have diameters of this magnitude or greater. If the smallest dimension is less, the molecule appears not to be retained in the blood stream, but to be lost through the kidney. Among molecules of the same diameter, where loss occurs it appears to be inversely related to the length of the molecule. This should apply not only to rod-shaped molecules of diameters of the order of 20 Ångströms or less, <sup>\* 16</sup> but also to the plasma proteins under pathologic conditions. Among plasma proteins with roughly the same diameter, of 38 Ångströms, the  $\beta_1$ -globulins which combine and transport copper and iron, have been estimated to have a length of 190 Ångströms, the  $\gamma$ -globulins, concerned with immunity, of 235 Ångströms, and fibrinogen, concerned with blood coagulation, of 700 Ångströms <sup>17</sup>

The viscosity of proteins depends, of course, not upon their size but upon their asymmetry. Thus fibrinogen, the most asymmetrical of the plasma proteins has an intrinsic viscosity six times that of albumin. Were it present in the plasma in large amounts, instead of to but 4 per cent, it would impose considerable burden upon the circulation. Serum albumin is far more symmetrical, a 25 per cent solution being isoviscous with blood <sup>18, 19</sup>

The albumins are, moreover, the most stable, the smallest and the most copious of the plasma proteins. Present in normal plasma to just over 50 per cent they are responsible for nearly 80 per cent of the colloid osmotic pressure which regulates the equilibrium in water and electrolytes between the plasma and the tissues, and

<sup>\*</sup> Characteristic of the various suggested blood substitutes which were examined but not recommended to the Armed Forces.



thus play the major role in maintaining the volume of the blood upon which normal circulation depends<sup>16-20</sup>

The molecular size and shape of proteins, once they are separated as homogeneous chemical components, may be estimated by measurements of osmotic pressure and of viscosity. The ultracentrifuge renders it possible, however, to distinguish proteins of different molecular dimensions even in so complex a mixture as plasma. The constant defining the speed of motion of a protein in the field of an ultracentrifuge, for the development of which we are indebted to Svedberg,<sup>21</sup> has revealed proteins sedimenting in plasma with four very different velocities.<sup>22-24</sup> Those sedimenting with the smallest velocity include the albumins and certain globulins. The other globulins which have been separated and fibrinogen sediment with other velocities. However, ultracentrifugal analysis does not permit us to distinguish a large number of components in the plasma.

*Electrophoretic Mobilities of Plasma Proteins* Solubility studies during the nineteenth century indicated that there were many globulins and classified them as euglobulin or pseudoglobulin, depending upon their insolubility or solubility, in the absence of salt, or in concentrated salt solutions. Electrophoretic analysis, refined by Tiselius, distinguished globulins in terms of their mobility in an electric field and designated them  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins.<sup>25-27</sup> Better resolution by the optical system employed in the analysis has revealed more than one  $\alpha$ -, more than one  $\beta$ -, and more than one  $\gamma$ -globulin. Two  $\alpha$ -globulins,  $\alpha_1$ - and  $\alpha_2$ -, two  $\beta$ -globulins,  $\beta_1$ - and  $\beta_2$ -, and two  $\gamma$ -globulins,  $\gamma_1$ - and  $\gamma_2$ -, the second one, in animals, sometimes termed a T-globulin, are now often designated. If we include the results of electrophoretic analyses the number of protein components of plasma recognized by physico-chemical means has thus increased to nine or more from the three recognized a century ago.

*Hormones* A very large number of protein components of the plasma has been postulated on the basis of physiologic or immunologic properties. Some of these have been concentrated and characterized. Others, like the hormones, by definition components of the blood, have rarely been separated from it, or even detected in it.

*Immunoproteins* Immunologic studies have led to the recognition and the study of complement and its components<sup>28-30</sup> and of a variety of antibodies.<sup>31</sup> Antibodies have been characterized, as euglobulins and as pseudoglobulins, in terms of their solubilities. Many, but not all, antibodies have been characterized as  $\gamma$ -globulins in terms of their electrophoretic mobilities. Among  $\gamma$ -globulins some have been described of very high molecular weight, others of molecular weights of the order of 156,000. For the most part, however, these immuno-chemical studies have not led to the isolation of pure antibodies of which there could conceivably be as many as the antigens which have led to the production by the body of specific antibodies.

*Enzymes* Prothrombin is presumably present in the body in but small amount and the action of thrombin is now generally regarded as enzymatic. The presence of a large number of other enzymes has since been demonstrated by virtue of their specific interactions. Thus there is the proteolytic, fibrinolytic, enzyme now called plasmin, a well-defined serum esterase, two phosphatases, a lipase, an amylase, and a number of peptidases, among them hypertensinase. Most of these substances

are far better known in terms of their chemical interactions than of their molecular properties

*Lipoproteins* Recent investigations have demonstrated the presence of different lipoproteins in the plasma, moving in the electric field respectively with the mobilities of  $\alpha_1$ - and  $\beta_1$ -globulins. One of these lipoproteins is an asymmetric molecule with a molecular weight of roughly 200,000. Another is a large spherical lipoprotein with a molecular weight of over a million.<sup>17</sup> These lipoproteins are noteworthy both because of their physical properties and because they render soluble such water-insoluble lipids as cholesterol, carotene and the steroids and because the specificity of their interactions is such that one of the estrogen hormones, estriol, has been found to be combined not with all, but only with one of these lipoproteins, the large spherical  $\beta_1$ -lipoprotein.<sup>32</sup>

*Albumins* Until recently the greatest emphasis has been upon the osmotic function of the albumins in maintaining the equilibrium between water and electrolytes in the blood and in the tissues. The development of normal human serum albumin for use in military medicine, for the treatment of shock, burns and hypoproteinemia, depended upon the molecular properties of albumin. However, as Bennhold<sup>33</sup> and later investigators<sup>34</sup> suggested, albumins interact with a variety of smaller molecules, notably with nonpolar anions such as aliphatic fatty acids<sup>35-37</sup> and are presumably responsible for their transport in the blood stream. Albumins also interact with a variety of dyes<sup>33</sup> including Evan's blue, often used in estimating blood volume<sup>38</sup> with naphthoquinones such as those developed as antimalarials<sup>39</sup> and with a variety of other dyes.<sup>40-42</sup> Albumins also combine with a variety of drugs, such as atabrin, neosalvarsan,<sup>33</sup> and digitoxin,<sup>43</sup> mercurials<sup>44</sup> and sulfa drugs.<sup>45, 46</sup>

*Crystallized Human Serum Albumins* Not all of the properties that have been ascribed to the albumins are due to these molecules. In order to demonstrate this, it was necessary to prepare highly purified crystallized human serum albumins.<sup>47</sup>

Human serum albumins that had been crystallized by earlier methods were demonstrated to contain over 2 per cent of long chain fatty acid.<sup>48</sup> The albumins that we have crystallized, in very satisfactory yield, from alcohol-water mixtures of defined pH and ionic strength at low temperatures also contained fatty acid, but the amounts present were far smaller, of the order of one mole stearic or oleic acid per mole of albumin. The fatty acid appeared to form an integral part of the crystal structure and, in fact, crystallization appears to be greatly aided by the presence of such amounts of fatty acid and by the addition of higher alcohols such as n-decanol. The amounts of the alcohol that have been found useful and with which the albumins combine range from two to ten moles per mole. Crystallized with the aid of such reagents, albumins can be recrystallized under a variety of physical chemical conditions and in a variety of crystal forms.

The resolution of the various albumins that crystallize together required a more specific method of crystallization in order to yield chemical individuals. Conditions for crystallizing horse serum albumin of constant solubility had been determined in our laboratory before the war by McMeekin.<sup>49</sup> This method has thus far not been found effective for crystallizing a fraction of human serum albumin. However

a large fraction of the human serum albumins crystallized by the decanol method has been found by W L Hughes, Jr, to form a relatively insoluble crystalline mercury compound<sup>50</sup> The albumin separated in this way appears to be a chemical individual whose solubility behavior approximates to that of a simple chemical substance

Although serum albumins combine with a larger number of equivalents of mercury the amount with the albumin in the solid phase of crystals precipitated in this way is but one-half a mole of mercury per mole albumin That is to say, each mole of mercury appears to be combined with two albumin molecules in the solid state<sup>50</sup> In solution, however, this complex dissociates Albumin of double molecular weight has been detected in the ultracentrifuge and reconverted to normal size either by the removal, or the addition of larger amounts, of mercury

*Pigment Proteins* Serum albumin had previously been reported to combine with hematin and with bilirubin<sup>53</sup> Upon adequate recrystallization, the amount of both diminish until they can no longer be readily detected spectrophotometrically Upon equilibrating such pure serum albumin with these substances, however, combination can be demonstrated and quantitatively estimated Albumin which we have recrystallized has been studied in equilibrium with hematin<sup>51</sup> and with bilirubin<sup>52</sup> At alkaline reaction albumin combines with as much as three moles bilirubin per mole albumin At acid reactions, however, the bilirubin is free and can be removed by dialysis

Bilirubin is also a component of a true pigment protein of the blood stream, normally present to less than a tenth of a per cent of the plasma proteins This bilirubin pigment protein interacts strongly and is, therefore, separated with difficulty from the 50 per cent of albumin in plasma It has now been separated, however, and alone of adequately purified plasma proteins, gives the indirect van den Bergh reaction<sup>53</sup> Another pigment protein responsible for a very characteristic blue-green color does not give this reaction and although often found associated with crude albumin preparations is a globulin concentrated in our system of fractionation in Fraction IV-1, whereas the yellow pigment, due to bilirubin, is concentrated in Fraction V-1

*Iodoproteins* Iodine combines with essentially all proteins, entering the phenol ring to form diiodotyrosine and also entering the imidazol ring Albumin rich in iodine has been prepared and crystallized by Salter from horse serum<sup>54</sup> In studying the distribution of iodine in the human plasma fractions that we have separated some has always been found with the albumin, some however, has been found in Fraction IV-6 The further study of the iodoprotein in these fractions should reveal more regarding the nature of the plasma molecules of which it is a part

*Metal-Combining Proteins* It has long been known that copper, iron and zinc are combined by plasma protein The separation of the plasma proteins into fractions in which are concentrated the molecules responsible for specific interactions has yielded, in Fraction IV-7, the  $\beta_1$ -globulin responsible for the combination and transport of copper and iron and perhaps of zinc in the plasma The close interrelation of the copper and iron in plasma had been noted in clinical studies<sup>55-57</sup> The combination of a component of plasma with iron was noted in connection with

TABLE 1. *Protein Components of Human Plasma Separated and Concentrated in Dense Fractions*

Fraction Component	Estimated Amount in 100 g. Plasma Protein	Concentrated in Fraction	Approximate Isoelectric Point	Specific Chemical Interaction
Fibrinogen	4	I-2	5.3	Thrombin
Non-clottable protein insoluble at low temperature	0.15	I-1		
Antihemophilic globulin*		I		
Antibody $\gamma$ globulins	11			
Diphtheria antibodies*	(0.01)			
Measles antibodies*				
Mumps antibodies*				
Streptococci antitoxin*		II	7.3	Antigens
Influenza antibodies*				
Pertussis antibodies*				
Typhoid H agglutinins*				
Antibody euglobulins		III-1	6.3	Antigens
Typhoid O agglutinins*				
Isogglutinins	(0.03)	III-1	6.3	Incompatible Red Blood cells
Anti A, anti B*				
Anti Rh antibodies*				
Complement components				
C'1	0.4	III-2†		Antigen-antibody complex
C'2		IV†		
Enzyme precursors				
Prothrombin	0.3	III-2		Thromboplastin
Plasminogen		III-3		Streptokinase
Serum enzymes				
Thrombin*		III-2	4.8	Fibrinogen
Plasmin*		III-3		Proteins
Amylase*				Starch
Lipase*				Lipid
Peptidase*		IV		L-Leucylglycylglycine
Phosphatase* (alkaline)		IV†		Phosphoric acid monoesters
Esterase*	0.02	IV-6	4.5	Acetylcholine, ethylbutyrate
Metal-combining $\beta_1$ -Pseudoglobulin crystallized	2.5	IV-7	5.6	Iron and copper
High molecular weight $\beta_1$ -globulins (lipid-poor)				
S=7	2	III-0		
S=20	1	III-0		
Iodoprotein*†		IV-6		

TABLE 1—Continued

Protein Component	Estimated Amount in 100 g Plasma Protein	Concentrated in Fraction§	Approximate Isoelectric Point	Specific Chemical Interaction
	grams			
Thyrotropic hormone*		IV-4		
Glycoproteins				
$\alpha$ -Glyco pseudoglobulin	0.7	IV-6	4.9	
$\alpha_2$ Mucoid globulin	0.5	IV-6	4.9	
Lipoproteins				
$\beta_1$ 75% lipid containing "X" protein	5	III-0	5.6	Estriol, carotenoids, and other steroids
$\alpha_1$ 35% lipid-containing protein	3	IV-0	5.2	Steroids
Blue green pigment $\alpha$ -globulin		IV-2		
Bilirubin-containing $\alpha$ -globulin†	0.05	V-1	4.7	Diazo reaction
Albumin crystallized with mercury		V	4.9	Mercury, decanol
Albumin crystallized with decanol	50	V	4.9	Fatty acids, bile salts, many dyes and drugs

\* These components represent but small proportions of the fraction and subfraction, and their properties cannot, therefore, be deduced from those of the concentrates in which they have been separated.

† These components have not been tested for since revision of the fractionation process.

‡ Albumin binds more bilirubin than the bilirubin pigment globulin in Fraction V-1 and more indine than has been found in Fraction IV-6.

§ When purified chemical components have been separated from fractions they have not been given new fraction numbers. In that case, the fraction number refers to the starting material for the separation of the component.

bacterial studies<sup>58</sup> and has led to its identification in a plasma fraction, to its further purification and characterization, and recently to its crystallization in collaboration with Bernhard A. Koechlin,<sup>59-61</sup> in our laboratory. Physiologic studies of the role of the separated protein injected into man have begun<sup>62-64</sup> with a view to determining its function and possible value in therapy.

*Specificity of Chemical Interactions.* The multivariable method for the fractionation of plasma that we have developed not only separates protein components in terms of their molecular properties, but also in terms of the very specific chemical configurations upon which their interactions depend. Insofar as the system of fractionation is successful each protein component responsible for a specific chemical reaction and physiological function is separated from those of different properties and concentrated. Thus one fraction should be positive with respect to any test to which the whole plasma is positive, all others should be negative. The extent

to which this end has been accomplished may be demonstrated by the following studies upon protein interactions.\*

### 1. Interaction of Naphthaquinone (M1523†) Caprylate and Serum Albumin

#### Solutions

Five per cent solutions of the following proteins are used. Volume of each is 10 ml.

$\gamma$ -Globulin (Fraction II) in acetate buffer pH 4.5-12 = 0.05

$\beta_2$ -Globulin (Fraction IV-7) " = "

Albumin (Fraction V) " = "

Albumin (Fraction V) in caprylate " = "

An acetate buffer blank " = "

Albumin control in acetate buffer as above but with naphthoquinone added 15 minutes in advance of the demonstration time

#### Reagent

Naphthaquinone, M1523‡

#### Method of Demonstration

To beakers containing each of the above solutions a small amount of naphthaquinone is added, stirred, and let stand 10-20 minutes. A beautiful red color develops in the solution containing acetate, but is blocked in the albumin solution; control and caprylate solutions and blank give fainter colors.

### 2. Binding of Bile Salts by Serum Albumin

#### Solutions

Serum Albumin, 2.5 per cent solution

#### Reagents

Bile Salts, 2 per cent solution

Red cells as an indicator

0.15 M sodium chloride solution

3 *Binding of Copper and Iron by  $\beta_1$ -Globulin (Fraction IV-7)**Solutions*

2 per cent solutions of the following proteins are used Volume of each solution, 300 cc

Fibrinogen (Fraction I)	} All are dissolved in pH 8.5† $\Gamma/2 = 0.05$ barbiturate buffer
$\gamma$ -Globulin (Fraction II)	
$\beta_1$ -Globulin (Fraction IV-7)	
Albumin (Fraction V)	
$\beta_1$ -Globulin (Fraction IV-7) control*	

*Reagents*

1  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$

Four bottles each containing 0.027 Gm  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  dissolved in 20 cc  $\text{H}_2\text{O}$

2  $\text{FeSO}_4 (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$

Four bottles each containing 0.048 Gm  $\text{FeSO}_4 (\text{NH}_4)_2\text{SO}_4 \cdot 6 \text{H}_2\text{O}$  dissolved in 20 cc 0.001 M acetic acid

3 One bottle containing 27 cc 1 M acetic acid

4 One bottle containing 27 cc 1 M NaOH

*Method of Demonstration*

All solutions must be stirred during addition of reagents

1 *Copper Binding*

Add copper solution to all protein solutions  $\beta_1$ -Globulin is the only protein solution that will give a positive reaction. A yellow-green color develops in 3-5 minutes pH is about 8-8.2

2 *Iron Binding*

Add iron solution to all protein solutions  $\beta_1$ -Globulin again gives the only positive reaction. A red color develops in 3-5 minutes as the copper is replaced by the iron pH is about 8-8.2

3 *Splitting Iron off the Protein†*

Add the acetic acid. The protein solution regains its original appearance pH is about 4.3

4 *Recombining the Iron*

Add the sodium hydroxide. The red color reappears pH is about 8-8.2

*Note* The yellow-green color of the copper and the red color of the iron become somewhat more intense with time

4 *Modified van den Bergh Reaction<sup>53</sup> for Identification of Bilirubin**Solutions*

1 per cent solutions of the following plasma fractions are used Volume of each, 120 cc

Fraction II

Fraction IV-2

Fraction IV-7

Albumin

Fraction V-1-B

} All solutions are made up in pH = 8.6,  $\Gamma/2 = 0.025$  §barbiturate buffer

\* The reagents listed below are added to the control about 4-6 hours in advance of the demonstration time

† This pH is lowered slightly upon the addition of the copper and iron salts, due to acidity of the salts themselves, and because 0.001 M acetic acid is used to dissolve the  $\text{FeSO}_4 (\text{NH}_4)_2\text{SO}_4 \cdot 6 \text{H}_2\text{O}$

‡ It is necessary only to add acid and base to the  $\beta_1$ -Globulin solution since it alone gives a positive reaction to the iron

§ The ionic strength of Fraction II is adjusted to 0.04 with 1 M NaCl to keep it from precipitating on addition of reagents. If higher ionic strength for IV-2 and V-1-B is used, they will precipitate on adding reagents

*Reagents*

- 1 Solution A
  - 1 g Sulfanilic acid dissolved in 15 cc concentrated HCl and diluted to 1 liter with water
  - 2 Solution B
  - 0.5 per cent  $\text{NaNO}_2$
  - 3 Diazo reagent prepared fresh by adding solution B to solution A in the following ratio 0.3 cc B to 10 cc A
  - 4 Absolute methanol
- The reaction mixture to be added to the protein solutions is prepared about an hour before the demonstration, as follows
- Add 5.7 cc solution B to 190 cc solution A. Add 190 cc of this diazo reagent to 950 cc absolute methanol \*

*Method of Demonstration*

Add 180 cc of the above reaction mixture to each of the protein solutions with vigorous stirring. A precipitate may form, but will redissolve.

The V-1-B solution will develop a pink color almost instantaneously. The other solutions give a negative reaction.

*5 Coarse and Fine Types of Fibrin Clots†**Solutions*

A 2 per cent solution of Fraction I is prepared for this demonstration.

*Note* The Fraction I used is dried from the frozen state from a 2 per cent isotonic solution in sodium citrate, volume 300 cc and it is only necessary to add 300 cc distilled water to reconstitute it. pH = about 6.3-6.6.

After the Fraction I is dissolved it is clarified, by filtering successively through D-5 and D-5 Horman filter pads. These pads may be first washed with 0.1 M acetic acid, then with water to free them of acid.

After filtration the solution is divided into two 125 cc portions. One remains as it is at pH 6.3-6.6, the other is adjusted to about pH 8 by the addition of 18.8 cc 1 M  $\text{NaHCO}_3$  solution.

*Reagent*

The only reagent necessary is a thrombin solution made by dissolving two small bottles of thrombin (about 500 units each) in 50 cc 0.15 M NaCl solution.

*Note* If this solution is cloudy it is clarified by filtration through a small D 5 Horman filter pad.

*Method of Demonstration*

*Coarse Clot* To the pH 6.3 portion of Fraction I, 12.5 cc thrombin solution are added and stirred quickly to mix the two solutions.

In about 5 minutes a clot forms firmly enough to permit inversion of the container. This clot is opaque.

*Fine Clot* To the pH 8 portion, 12.5 cc thrombin solution are added and stirred quickly.

In about 5 minutes or slightly longer, a firm clot forms, so that the container can be inverted. This clot is clear. *Note* This clot forms a little more slowly than the coarse clot.

## SUMMARY

The chemical methods that have been developed for the separation, concentration and purification of the protein, glycoprotein, mucoprotein, and lipoprotein

\* Reaction mixture should not develop a color. If a color develops it is probably due to an impurity in one of the reagents. Therefore fresh reagents should be prepared.

† This demonstration depends upon the experiments of J. D. Ferry and P. R. Morrison.



components of any biologic system, by fractionation in alcohol-water mixtures controlled pH, salt, and protein concentration, at the subzero temperatures necessary to prevent denaturation, have thus far led to the recognition and concentration of over twenty-five different protein components of human plasma. These include albumins of more than one kind, immune globulins which differ in their physical properties and interactions with antigens, lipoproteins which differ in their physical properties and interactions with steroids, enzymes with proteolytic, peptidase, lipase, phosphatase and esterase activity, thrombin, fibrinogen, and the antihemophilic globulin concerned with blood coagulation, iodoprotein and the recently crystallized metal-combining protein which interacts with both copper and iron and is presumably concerned with transport in the plasma.

This number of plasma proteins is far greater than can be detected electrophoretically or in the ultracentrifuge. Chemical fractionation has yielded at least four  $\beta_1$ -globulins and at least two  $\alpha_1$ - and three  $\alpha_2$ -globulins. The  $\alpha_2$ -globulins include mucoprotein and glycoproteins of more than one kind, the  $\alpha_1$ -globulins, the bilirubin-containing globulin in Fraction V-1 and the lipoprotein in Fraction IV-1. The  $\beta_1$ -globulins include the carotene-rich euglobulin which combines with three times its weight of lipid as well as a high molecular weight lipid-free  $\beta_1$ -globulin, both of which are concentrated in Fraction III-o. Fraction III also contains  $\beta_1$ -globulins of different molecular properties. The iron-binding component of the plasma, crystallized from fraction IV-7, is a lipid-free  $\beta_1$ -globulin and is more closely related to the albumins than to other globulins from the point of view of osmotic activity. Electrophoretically indistinguishable, these different  $\beta_1$ -globulins have no other common property. The lipid-binding plasma component is a  $\beta_1$ -euglobulin, the iron-binding  $\beta_1$ -component a pseudoglobulin. They differ in size, shape, in solubility, in chemical composition and interaction, and in physiological function.

The separation and concentration of the various proteins of human plasma was undertaken during this war in order to render as many as possible available as therapeutic agents and thus to increase our knowledge and control of the composition of the blood in health and in disease. Many more have been separated and are being studied chemically than have thus far been brought to clinical trial. Their study renders possible the further investigation of the chemical specificity of the interactions of the plasma proteins, which are responsible for many of the tests that have in the past, or may in the future, prove of value in the clinic in the study of pathological sera and the understanding of the specific protein component that is either elevated or deficient in this condition.

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# THE SPAN OF LIFE OF THE RED BLOOD CELL A RÉSUMÉ

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THE QUESTION of the life span of the red blood cell has been an open one for the past hundred years. Beginning with naive attempts to determine the duration of the corpuscle in the circulation by transfusions of foreign nucleated cells into the mammalian blood stream,<sup>1</sup> the quest continues as of November 1946 with the most modern biologic tool, the isotope, and with full benefit of higher mathematics in the analysis of the data collected. A solution at last seems to be in sight.

The fact that the mammalian red blood corpuscle is an incomplete cell, which has lost its nucleus, together with the fact that it is of necessity subjected to the stress of contortion as it passes through the capillaries, has made its ephemeral existence an acceptable hypothesis. To anyone who has had the privilege of seeing the beautiful moving picture made by Professor August Krogh showing the opening and contracting of the capillaries and of the red blood cells being crowded through them, it is amazing that the frail erythrocyte should survive for any great length of time.

Two schools of thought have arisen concerning this subject. One is influenced by the seeming fragility of the corpuscle irreconcilable with any long buffeting in the circulation. Its members still accept the earlier work upon the rate of bile pigment excretion which argues for a short life of the cell.<sup>2</sup> The chief protagonist of this school is Dr. Raphael Isaacs. His expressed opinion as of 1938 was as follows: "As the erythrocytes in mammals are non-nucleated it is evidently impossible for repair and metabolic nutrition to be a part of their normal physiology. Their life duration must be limited and is probably shorter than most of the fixed tissue cells, but the exact or even approximate length of service is not known."<sup>3</sup> This opinion is still acceptable to many authorities in the field of blood physiology and has appeared in some of the postwar textbooks, in which the physiology of blood destruction is discussed. For instance, in "An Integrated Practice of Medicine"<sup>4</sup> (1946), edited by Harold Thomas Hyman, we find the following statement on page 1038: "The life of the normal erythrocyte is very limited and probably does not exceed a span of more than six weeks."

The other school bases its judgment upon certain technics which tag the corpuscle, either directly or indirectly, and enable the observer to follow it until it is eliminated. The members of this school accept, notwithstanding its seeming improbability, a life span of approximately 120 days for the red cell in the normal body.

## THE METABOLISM OF THE MAMMALIAN RED BLOOD CELL

Since this supposed frailty of the non-nucleated erythrocyte has played so great a part in our thinking concerning the span of life of the red blood cell it would

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seem well to examine the evidence which concerns the position, from the point of view of metabolism, which the red cell holds in the society of cells which compose the mammalian organism

In the first place, although the nucleus is necessary for cell division and reproduction, it is not necessary for the continued life of the cytoplasm. This has been beautifully demonstrated by Dr. Chambers, especially in his work upon the enucleated amoeba, which survives for a long time, forming contractile and gastric vacuoles. The nucleus is not, as one was inclined to think in one's high school days, the brain which directs the metabolic activities of the cell. The cytoplasm also has its enzymes by which its metabolic activities are accomplished. One thinks of the various components of the cell as being produced *in situ* from previous material of the same kind. For instance, botanists have long regarded the plastid, that component of the cytoplasm in which starch is produced when the leaf is illuminated, as being capable of reproducing itself and of passing on as such when cell division occurs.<sup>5</sup> In the recent conference on the chemistry and physiology of growth held at Princeton, the question was proposed as to the passivity of the cytoplasm and attention was called to self-perpetuating cytoplasmic entities in lower forms which could be passed from the cytoplasm of one cell to another, dependent to some extent upon the genes of the host.<sup>6</sup> In line with this are recent disclosures by way of the isotope. To quote Addison Gulick: "Studies with radioactive nitrogen have demonstrated that proteins in the living cell are constantly undergoing exchanges of their amino acid residues. Thus, even after it is completely synthesized, the protein molecule does not maintain its absolute identity of material, but merely a steady state with like configuration of like materials."<sup>7</sup> The same author mentions the fact that the cytoplasm has its own nucleoprotein, which is different from the nucleoprotein of the nucleus, but is of the same type as that found in viruses, or at least in the tobacco virus. And the tobacco virus is not only capable of maintaining itself, but, given the cooperation of a suitable cell, of very extensively reproducing itself. Red cells are reported to contain nucleoproteins. They also contain catalysts such as phosphatase, carbonic anhydrase, coenzyme, choline esterase, catalase, urease, lipase. Barron<sup>8</sup> quotes some work indicating that red cells can synthesize flavin adenine-dinucleotide from riboflavin *in vitro* and *in vivo*. It would seem then that the long cherished conception of the histologically minded hematologists that the red cell is a passive hemoglobin containing sac with little other content, will have to be modified.

We have several indirect points of evidence to the effect that the red cell may be able to repair itself. For instance, a small amount of glucose, the prime source of metabolic energy, aids in preventing deterioration of drawn blood. Maizels has shown that glucose inhibits the breakdown of organic phosphates in the stored cells.<sup>9</sup> Maizels and Paterson showed that stored cells that had taken up sodium at the expense of their normal potassium, although they still possessed this excess after half an hour in the blood stream, lost it in twenty-four hours against the steep concentration gradient produced by the content of the patient's serum. They consider that the cells have been "reconditioned" in a more complex manner than would be accounted for by a simple physical process.<sup>10</sup> There is also the loss of the

specific polysaccharides constituting the a and b antigens, which takes place when cells are stored under the stabilizing influence of a lowered temperature and removed from the strain of the circulation. Whereas under the stress of the circulation the high degree of agglutinability is maintained. This would suggest that in vivo the corpuscles have capacity to replace the loss of these specific polysaccharides.

The very low metabolism found by Warburg, at least in the corpuscles of man, is rather against the idea of the synthetic repair of the red cell in the blood stream, since energy is required for the building of protein and of the polysaccharides.<sup>11</sup> Ramsey and Warren find the respiratory rate of red cells to be comparable to the rate of other resting tissues ( $30-70 \text{ mm}^3/\text{Gm/hr}$ ).<sup>12</sup> G. P. Wright finds that normal orthochromatic erythrocytes have a negligible respiration, if any, but that oxidation increases with increases in reticulocytes and is in proportion to their number present.<sup>13</sup> Similar findings had previously been reported by Barer, Needle and Baldrige.<sup>14</sup> The argument in favor of any metabolic repair in vivo would be in a bad way if it were not for the work of Harrop and Barron who showed that non-nucleated erythrocytes respire to an extent comparable to the respiration of other tissues in the presence of a hydrogen acceptor. They used methylene blue.<sup>15</sup> Following this, Michaelis and Salmon found in aqueous extracts of the body tissues, notably the liver, something which acted in a manner similar to methylene blue and raised the respiratory rate of the red blood cells to the level of tissue cells.<sup>16</sup> It would seem almost certain, then, that in vivo the metabolism of the red cell is such that the cell would have the energy necessary to repair its own deteriorations.

### THE STUDY OF TRANSFUSIONS AND HEMORRHAGE

The first attempts to determine the life of the erythrocyte, that from the point of view of our present knowledge must be considered seriously, were the studies of transfusions or of hemorrhage using changes in the total blood count as the indicator. Hunter,<sup>17</sup> in a report before the Royal Society of Edinburgh in 1885 of his own work in this line and of that which preceded his, gives the results obtained by Ward-Miller, Quinck, and von Ott. Ward-Miller transfused dogs and produced a plethora. After two or three days he judged that the number of corpuscles responded to the number transfused plus the original count. After a few weeks the count had returned to its first level. Hunter, in order to avoid the abnormal condition of plethora, injected blood intraperitoneally, von Ott removed large amounts of blood, injected defibrinated blood and observed the time taken for the total count to drop to a minimum. The period of observable change indicated by this work was between fourteen and twenty-six days.

Various modifications of the above type of experimentation have continued to date. In 1934, Escobar and Baldwin introduced a new factor in that they produced the plethora by exposure to low oxygen pressure.<sup>18</sup> They also measured plasma volume. They did not count reticulocytes, which Rous and Robinson<sup>19, 20</sup> found reduced to zero in their studies of increase in fragmented corpuscles produced with plethora. They did not allow the cell count to increase under oxygen need beyond a certain arbitrary amount because they said, "If the cell count is too great the

compensatory erythro-destroying mechanism begins to act and the supernumerary cells under observation may not live out their allotted normal life span." The results of these workers are in good agreement with other work in which this general method has been used. In rats the count returned to normal in from twelve to eighteen days, in the dog from sixteen to twenty-three days, in man from eighteen to thirty days.

In all of this work which deals with the whole blood there are three underlying factors, and probably a fourth, two of which are quantitatively indeterminate and therefore vitiate any quantitative results. The quantitatively indeterminate factors are blood destruction and blood production. In the reticulocyte count we have a qualitative index of blood production, but there is no way in which the number of reticulocytes can be interpreted in terms of the number of red cells that are being produced. The length of time that an increased count remains above normal could be taken as an index of the length of time that the cells producing this increased count remained in the circulation, only if we were certain that no change had taken place in the relative rate of production and destruction which maintained the original cell count. This we cannot assume. In fact, we have evidence in the work of Rous and Robertson<sup>21</sup> that plethora causes a reduced production, as shown by absence of reticulocytes and by a reduction in the count of cells in the bone marrow, and also an increased destruction, as shown by the increase of schizocytes in the spleen. Both increased destruction and decreased production would cause the count to return to normal without the necessary destruction of the cells introduced to cause the excess counts, especially if the excess cells were the youngest cells in the community, as would be the case in the experiment of Escobar and Baldwin. The duration of the plethoric count would always be shorter than the life of the cells which produced it.

In 1930 Eaton and Damren made an interesting contribution to the problem.<sup>22</sup> They subjected rabbits and dogs to fairly severe hemorrhage and studied the appearance of reticulocytes. The curve of their appearance showed marked crests which gradually subsided through five or six cycles. The interval between crests in the dogs' curves was approximately sixteen days and that of the rabbits eight and one-half days. They infer that the blood which is produced in the first crest is destroyed and its destruction is the stimulus for the second crest, and so forth.

These data would be strong evidence in favor of the shorter term of life of the red cell, if there were not some indication that activities of the organism do not proceed at a constant rate, but are subject to more or less regular fluctuations. From this point of view these data could be explained as magnifications of these tendencies to periodic increases in growth activity caused by the stimulus of increased need. This tendency to rhythmic fluctuations in the anabolic and catabolic activities of the organism, which appear to become more evident under stress, needs to be recognized clearly in attempting to evaluate the evidence concerning the length of life of the red cell.

Ashby in 1921 reported that "Group O corpuscles transfused into a recipient with agglutinable corpuscles disappeared in steplike intervals that might be from two to four weeks in span."<sup>23</sup> Wearn, Warren and Ames using the same techni-



failed to observe these abrupt changes, but they admitted that their observations may not have been sufficiently closely spaced to catch them <sup>24</sup> Of course there are many rhythmic phenomena which have been studied in relation to the menstrual cycle, but there is also evidence that such rhythms occur independently They may be seen in weight curves in persons on a reducing diet The cross striations on the fingernails have been attributed to such fluctuations in growth Upon measuring the growth of a series of fingernails through several weeks, I have found such fluctuations at intervals of from twelve to fourteen days <sup>25</sup> Schultz, in a study of fetal growth in man, states that the rates of growth of different parts of the body are frequently found to alternate during fetal life which indicates a certain fluctuation in the rate of growth " (p 391) <sup>26</sup> One of the most careful studies of these fluctuations was made by Broun, McMaster and Rous <sup>27 28</sup> They used this phenomenon in their study of the relation between blood destruction and the output of bile pigment They describe it as slow, wavelike changes in hemoglobin content and bile pigment output with often as much as a fortnight elapsing between crest and crest If we are willing to accept the longer life of the red blood cell, then the findings of Eaton and Damren also become evidence for a periodicity in the activity of the hemopoietic system

#### BILE PIGMENT METABOLISM AND THE SPAN OF LIFE OF THE RED BLOOD CELL

Since bile pigment is derived from hemoglobin it is inevitable that measurements of bile pigment excretion will have played a large role in our estimations in the life of the red cell

The earlier conceptions of bile pigment as derived exclusively from the breakdown of hemoglobin and as being excreted in its entirety, give the red cell a short life, when calculated on the basis of the known quantitative relationship between hemoglobin and bile pigment Wilber's hypothesis of a conservation of the pyrrole complex for the formation of fresh hemoglobin by reabsorption from the intestine would still further reduce the figure for life span of the red cell It was not until Whipple and Hooper introduced the conception that bile pigment might be derived from other sources than hemoglobin that evidence of a longer life of the red cell became tenable

Following the earlier work, in which calculations were based upon a direct relationship between bile pigment excreted and hemoglobin destroyed, Eppinger in 1913<sup>28</sup> Wilber and Addis<sup>29</sup> in 1914 and Addis<sup>2</sup> in 1915 emphasized the probability that bile pigment was reabsorbed from the intestine and used for the formation of hemoglobin as the needs of the organism demanded This theory led to a great deal of experimental work, some of which appeared to support it and some to disprove it

In 1917 Hooper and Whipple<sup>30</sup> published the results of some work on dogs which, they considered, indicated that bile pigment was not absorbed from the intestine They studied dogs with biliary fistulas to determine the normal rate of bile pigment excretion, after which they injected from 100 to 300 cc of fresh bile by stomach tube They determined the rate of excretion of bile pigment for six hours and in some cases for fifteen hours afterwards They found no significant increase

In 1923 Broun, McMaster and Rous<sup>31</sup> repeated this work with an improved technic for collecting the bile which enabled them continuously to obtain the whole twenty-four hour specimen. They worked with dogs and part of their experiments consisted in the injection of sheep bile which contained cholehematin, a substance not found in dog bile. They found this in the dog's biliary secretion and considered this partial evidence that the sheep bile had been taken up from the intestine, although they admitted that cholehematin was an extrinsic substance obtained from the green food of the herbivorous animal. They also fed dog-bile, but in this work their results were not consistent. In some instances they considered that their figures indicated an increase in the output from the biliary collection upon feeding bile, in others no increase was shown. They considered that on the whole their results indicated an enterohepatic circulation of bile pigment.

In 1926 Bollman, Sheard and Mann<sup>32</sup> brought a fresh technic to the solution of the question. They compared the bilirubin contents of the venous and arterial blood supply of various organs, including the intestine. They used fasting animals, animals on a mixed diet of meat, bread, milk and syrup, animals given a feeding of cream and egg yolk, and animals given fresh dog bile in amounts of from 100 to 200 cc, injected into the duodenum, the jejunum and the ileum. Comparisons were made of the bilirubin contents of the blood from the mesenteric veins and arterial blood at periods of from thirty minutes to three hours after administration of the bile, and at suitable periods in the fed animals. They had previously found that blood samples withdrawn simulatenously from different arteries of the body contain identical amounts of bilirubin. The bilirubin content of blood from venous sources taken at the same time is greater than or identical with that of arterial blood, depending upon the areas drained by the veins. The findings indicated that bilirubin was added to the blood in the spleen, the bone marrow and the liver. With the same technic they were unable to detect any increase in the blood returning from the kidney, skeletal muscles or the intestine. They conclude that since their method was sufficiently sensitive to detect the addition of bilirubin to the blood as it passes through any of the sites of bilirubin formation, no intestinal absorption occurs under the conditions of their experiment.

Probably the latest contribution to this problem as to whether the bile pigment is absorbed and reutilized comes from some work by Shemin and Rittenburg<sup>33</sup> using the isotope  $N^{15}$ . The isotope was incorporated into glycine which had in previous work been shown to be the nitrogenous precursor of the protoporphyrin of hemoglobin. When the isotope containing glycine was fed to man the  $N^{15}$  content of the hemoglobin rose sharply, stayed on a level and then fell abruptly to base. The flatness of the curve indicates that there was no reutilization of the labeled nitrogen for hemoglobin formation.

Having apparently disposed of the question of bile pigment reutilization, we come to the even more important one in our assay of the life of the red cell, that of whether or not the bile pigment complex has other sources than hemoglobin.

In 1916 Whipple and Hooper<sup>34</sup> reported that bile pigment excretion was increased with carbohydrate feeding. By 1922 the studies on the effect of food had been greatly extended by the workers constituting this group. It was shown that

red meat, cooked liver, hemoglobin and butter fat had a positive effect upon production of hemoglobin or bile pigment. Next came spinach, full diets of common grain foods and milk. Chlorophyll, clams, onions, lard and codliver oil were inert. At this time Whipple<sup>36</sup> formulated the conception of a bile pigment complex which would be an intermediary stage in the development of bile pigment from hemoglobin and would be utilized according to the needs of the organism. He considers that bile pigment output is a result of functional activity of the liver and not solely the result of the passive elimination of defunct hemoglobin. At the time he characterized pernicious anemia as a disease which showed abnormal tendency to pigment production rather than to cell destruction. Preceding this, Ashby, due to a failure to find any increase in destruction of transfused red cells in that disease, had suggested that a retardation in the maturation of the red cells in the bone marrow might account for the accumulations of iron because of non-utilization and that the physiologic stimulus of the anemia might account for the extension of the active bone marrow and the increase in bile pigment production.

In 1923, Rous, Brown and McMaster<sup>37</sup> published a series of papers on studies of total bile, using their technic of continuous bile pigment collection. One of these papers was devoted to the question of the relation of carbohydrates to the output of bile pigment. They considered that their results did not substantiate those of Hooper and Whipple. They found an increase in bile pigment production upon carbohydrate feeding followed by a compensatory decrease. They explained this result on the basis of a temporary hastening of the evacuation of the bile pigment from the liver due to deposits of glycogen. In a paper on the relation between blood destruction and the output of bile pigment they described work in which dogs had been intubated with the total removal of bile for periods of three months.<sup>38</sup> A secondary anemia developed which showed intercurrent fluctuation accompanied by similar fluctuations in the bile pigment output. The relations between these fluctuations were studied. In addition, some of the dogs were subjected to treadmill exercise and to transfusion of citrated blood. The authors find that the output of bile pigment is not fully reflected in the fall in hemoglobin, which they interpret as blood destruction. They attribute this to a process of pigment conservation which varies in proportion to the body need. They add that "the destruction finds expression in terms of bile pigment and practically at once, and the data support the conception that bilirubin has no other source besides the hemoglobin of destroyed blood."

In the evaluation of all such data as the above we have certain inherent difficulties. Bile pigment can be measured, but blood destruction cannot. The blood count is the resultant of two unknowns—the rate of blood production and the rate of blood destruction. With a decrease in blood production the total red cell count would go down without any change in the rate of blood destruction. If we interpret these increases in bile pigment production which Rous and his co-workers have found associated with the falls in circulating hemoglobin as part of a stimulus to production of the pyrrole complex in answer to the need, they become equally potent arguments for the extra-hemoglobin source of bile pigment.

It would appear that Hawkins and Whipple<sup>39</sup> have finally settled the question

by pinning one of the unknown factors, blood production. They did this by removing within a short space of time enormous amounts of blood from dogs. Upon regeneration of the red cells they had a population of corpuscles that were of practically the same age. They varied the procedure by destroying the blood with acetyl phenylhydrazine to avoid removal of material that might be useful in rebuilding corpuscles. The bile pigment output changed from 125 mg per day to 58 mg and remained at this low level because the population of the blood stream was preponderantly young cells that were not being destroyed. After approximately four months the bile pigment rose to a high level. This was interpreted as being the result of the eventual death of the cells that had been formed after the massive hemorrhages. The life span was estimated at 133 days.

### THE USE OF DIFFERENTIAL AGGLUTINATION

After the establishment of the human blood groups by Landsteiner, followed by Jansky and Moss, much clinical experience was accumulated with reference to the blood transfusion. By 1918 the question was in debate by the medical profession as to whether or not transfused blood existed in the circulation for even the twenty-two days suggested by the work summarized by Hunter, or, whether it was removed within a few days and any favorable clinical results were due to the supply of raw material from which new cells could be formed more readily.

In 1911 an article by Charles Todd and R. G. White<sup>40</sup> had appeared in the proceedings of the Royal Society, 'On the fate of the red blood corpuscles when injected into an animal of the same species'. The authors used a highly polyvalent isohemolytic cattle serum produced by injection of cattle with the blood of other individuals. By this technic they had previously reported a high degree of individuality in cattle similar to that found by Ehrlich in goats. This serum was exhausted for the antihemolysins of the corpuscles to be studied. It was used to separate transfused blood from the blood of the recipient. The transfused blood was found to disappear in the course of a few days. The authors found that the injected corpuscles are treated by their host as foreign, and in fact act as antigens, and give rise to the formation of corresponding antibodies in accordance with the ordinary laws of immunity.

In 1918, Ashby<sup>41</sup> at the Mayo Clinic, taking advantage of the difference in agglutinability of the recipient and donor cells in instances of transfusion with the universal donor," published the result of the study of 3 cases in which it was claimed that the transfused blood stayed in the circulation for considerable lengths of time, time enough to produce beneficial results due to functioning cells. Because of the rapid turnover of patients at the Mayo Clinic it was difficult to follow the full span of life of the transfused blood, and nothing was postulated as to this point. Of the 2 cases reported, which remained under observation for forty days, in one, that of a woman who had had a total hysterectomy, the transfused blood had come to base, while in the other, a man who was transfused for simple hemorrhage, there was little sign of disappearance of the transfused blood at the end of this time. Included in this first report were studies checking the quantitative validity of the technic with *in vitro* mixtures of bloods and certain points necessary for

satisfactory results were stressed. There were also included 10 cases studied for only a short time, in which the data indicated a common factor relating the amount of blood transfused, the body weight of the patient and the number of cells found in the blood after transfusion attributable to the transfused blood, as determined by differential agglutination. Such data were subsequently used in the study of blood volume.

Three papers by the same author followed this initial report.<sup>42 23 36</sup> They gave data on approximately 40 cases. Many of these patients were suffering from fatal illnesses. Although the longer life of the corpuscle was amply demonstrated in a few patients who were in comparatively normal health, attention was called to the great irregularity in the time taken for elimination of the transfused cells in a group of patients in which it had been possible to follow the count to its extinction. These survival periods ranged from 30 to 110 days. It was argued that transfused bloods, having been taken from normal donors, had equal capacities to survive and that the differences in survival periods were due to an activity of the organism receiving the blood. This argument was offered in conjunction with the data in connection with certain cases in which periodic steplike decreases in the count were seen. The capacity for the long survival of the red cell in the blood stream, however, is seen. In 11 cases that were under observation for periods varying from 22 to 51 days during which no appreciable drop in the count of transfused blood took place, from the case of a man who was in good health who had been transfused for a simple hemorrhage and whose count was followed for 110 days when there was still evidence of some of the transfused blood, from cases of pernicious anemia in which there was evidence of survival of the last of the transfused corpuscles, which were presumably the youngest at the time of transfusion, 95 and 100 days after the transfusion had been given.

In 1922 Wearn, Warren and Ames,<sup>21</sup> using Ashby's technic, presented prolonged studies on 8 patients from the Medical Service of Peter Bent Brigham Hospital. Four were cases of primary anemia and four of secondary anemia due to nephritis. They report that 'the last of the transfused red blood cells disappeared from the circulation in from 59 to 113 days', 'No difference was noted in a series of observations in the duration of the stay of the transfused red blood corpuscles in the circulation between patients with primary and secondary anemia due to nephritis, and that 'in a single observation red blood corpuscles from a patient with pernicious anemia transfused into another patient with pernicious anemia, behaved as did corpuscles from normal donors'.

By 1926 several criticisms had been leveled against this apparently simple technic of Ashby, notably by Wildegans<sup>43</sup> and Gorl.<sup>44</sup> The criticisms were due to an inability to get quantitative separation of in vitro mixtures of agglutinable and nonagglutinable blood as had been reported by Ashby in the original description of the technic. These failures to repeat the results of Ashby were probably due to the use of serum of insufficient agglutinating strength or to the use of hemolytic serum, both of which were warned against in Ashby's original description of the technic. A possible cause, also, would be insufficient agitation during the initial period of agglutination. The technic was not acquired by these workers.

The most serious criticism of the findings of Ashby and of Wearn, Warren and Ames, indicating a prolonged life for the red cell in the blood stream, came from Isaacs, in 1924.<sup>45</sup> He reported "The use of agglutination in recognizing the cells of a donor in a mixture of two bloods in a transfused patient is of little value after the number of young cells reaches the number of unagglutinable cells, usually in from two to four days." This statement was based on work using a radical modification in technic, with no report of *in vitro* work to see whether or not the technic gave quantitative separation of an agglutinable from a nonagglutinable blood. The greater part of the work was done upon the blood of dogs in which animal there are not known to be strong isoagglutinins and for which the author gave no evidence that they exist. Work upon 2 human cases was reported, the results of which were entirely at variance with the results already reported with the Ashby technic on some 50 cases by Ashby and by Wearn, Warren and Ames.

Ashby immediately criticized this paper of Isaacs,<sup>46</sup> in the first place because the immature cells which Isaacs had claimed composed the unagglutinated cells were not found among the nonagglutinated cells by the Ashby technic. The same finding was reported in 1940 by Maizels and Paterson who say "If the unagglutinable cells are immature cells they should show a high retic count, higher than that of the whole blood, but the reverse is the case." In the second place the prolonged rise in the count of unagglutinable cells after a Group O transfusion and the lack of a rise after a like group transfusion reported by both Ashby and by Wearn, Warren and Ames was not explained by Isaacs. It was also pointed out that on the basis of the amount of blood given and the body weight of the two patients, the counts of the unagglutinable blood found by Isaacs subsequent to transfusion would indicate an impossible blood volume relationship, being in one case 25 per cent of the patient's body weight and in the other 75 per cent of the body weight. It was concluded that Isaacs had not accounted for the transfused blood by his technic.

Isaacs' results, however, were considered seriously by the group interested in the longevity of the blood cell and Isaacs, himself, reiterated them in a paper in *Physiologic Reviews* in 1937,<sup>48</sup> so that at the beginning of World War II when several English groups were studying the effect of storage upon survival of transfused blood for use at the front, Maizels and Paterson<sup>47</sup> undertook to check on the validity of Isaacs' claims. With reference to Isaacs' criticisms of the Ashby technic they state "It may be said at once that these criticisms are theoretical and unsupported by numerical data, however, since the cell agglutination technique is the only one which permits of a direct measure of cell survival the objections must be considered in detail. The arguments were approximately those used above except that in addition the M and N factors were used to separately agglutinate the donor and recipient cells."

By 1928, however, Landsteiner, Levine and Janes<sup>49</sup> had offered irrefutable disproof of Isaacs' contention for the short life of the cells by making use of their anti-M and N sera and agglutinating the transfused cells instead of the recipient's cells. They found clumps in the recipient's blood treated with their antiserum for seven weeks after transfusion, when they ceased to examine it. Later Wiener,<sup>50</sup> using

M antiserum with an M donor, reported that the life of the transfused cell probably averages between 80 and 120 days

The very considerable amount of work which was done at the beginning of the war, checking the various methods for preserving blood and the time allowable for storage, made use of the cell-agglutination technic. This work was carried out by the English Groups,<sup>47 51 52</sup> by Wiener and Schafer<sup>53</sup> in this country and by the Russians in checking their cadaver blood. This work all indicated the long survival of the transfused corpuscle.

#### DETERMINATION OF THE LIFE SPAN OF THE RED CELL BY USE OF THE ISOTOPE

The problems involved in tagging the red cell by use of the isotope and determining its longevity are: First, to introduce the isotope into the corpuscle in such a chemical combination that it remains in the corpuscle during its life, second, to choose an isotope of some element that will not be reutilized after the corpuscle has disintegrated, and last to choose an isotope that can be studied for a sufficient length of time.

The interesting phenomenon which the use of isotopes has emphasized is that although morphologically the cell and its constituents are intact, the reversibility, within the status of their equilibrium of certain cellular reactions, results in continuous degradation and resynthesis. An isotope introduced into a constituent of the cell which bore such a relationship to the metabolism of the cell would not indicate by its presence the life of the cell, but would merely be an index of the time relationship of the reaction in which the compound was involved.

On the other hand, if the isotope is some element which will be reutilized in the formation of new cells its presence will extend beyond the lifetime of the cells into which it was first introduced. It has long been considered probable that iron split off from hemoglobin upon the formation of protoporphyrin, is quite labile and is readily reutilized. In 1941, Cruz, Hahn and Bale,<sup>54</sup> working with radioactive iron, reduced the iron reserves of dogs by repeated hemorrhage and fed radioactive iron. This appeared in the blood with the rise of hemoglobin and stayed on a level for seventy-five days when the dogs were treated with acetylphenylhydrazine. The resultant drop in hemoglobin was paralleled by a drop in the radioactivity of the blood which rose to its original height with the regeneration of hemoglobin. The isotope level had not decreased 180 days later. The authors conclude that iron liberated from red cells is utilized readily and nearly quantitatively for the regeneration of hemoglobin in new red cells.

In 1946 Shemin and Rittenberg introduced the isotope  $N^{15}$  into several compounds which were of interest in their possible relationship to the synthesis of protoporphyrin of hemoglobin.<sup>55</sup> Glycine, proline and glutamic acid were chosen because there was a probability that they would be used in the formation of protoporphyrin, leucine was chosen as a representative  $\alpha$ -amino acid whose intact carbon chain was unlikely to be used for pyrrole synthesis, and ammonia was chosen to test the nonspecific utilization of nitrogen liberated by deamination of amino acids. These substances were fed to rats whose hemin was subsequently tested for the isotope. It was shown that glycine is a nitrogenous precursor of the

protoporphyrin of the hemoglobin of rats. The authors were of the opinion that the much smaller amount of  $N^{15}$  found in the hemin upon feeding of isotope proline, leucine, glutamic acid and ammonia is due to  $N^{15}$  enrichment, by the nitrogen of these compounds, of the body nitrogen from which the precursors of heme is synthesized rather than a direct utilization of these compounds.

Having established this fundamental point these workers proceeded to feed  $N^{15}$  containing glycine to a man and to study the shape of the curve indicating the appearance in the circulation of this isotope and its disappearance from the circulation. 'The values rose rapidly to a high level, remained practically constant for many weeks and then fell quite sharply to a very low level. This finding indicates that the heme is neither involved in the dynamic metabolic state nor reutilized for hemoglobin formation. On these grounds, the curve of  $N^{15}$  concentration of the heme versus time can form a basis for the average life span of the human red blood cell. This was found to be about 127 days.'<sup>33</sup>

### CONCLUSION

We come then, after following the evidence derived from three separate approaches, to the conclusion that the life span of the red cell approximates the dimension of 110 to 130 days under favorable conditions.

This does not mean, however, that for practical purposes a transfusion lasts for that length of time. The blood transfused consists of cells of all ages up to the full span of four months, and half of them are already half of this age when they are transfused. Neither does it mean that the span of life of the red cell is of this dimension under all circumstances. Hawkins and Whipple reported the figure of 133 days from work on normal dogs. The study of Shemin and Rittenberg using the isotope  $N^{15}$  in which they found the life span to be 127 days was made on a normal man. The subjects of transfusion, however, are usually far from normal, therefore the data derived from the cell-agglutination technic has to be considered in relation to the condition of the patient transfused. Ashby (1921), Wearn, Warren and Ames (1922), Wiener (1934), Mollison and Young (1940), and Callender, Powell and Wits (1945),<sup>56</sup> are agreed upon 120 days as an approach to an optimum length of life for the transfused red cell as indicated by the method of differential agglutination. Ashby (1921), and Wearn, Warren and Ames (1922), are agreed that in pernicious anemia the tenure of life of the red cell is of a similar dimension. But there are other conditions in which the life span of the transfused blood and probably, also, of the patient's own blood, is of much shorter duration.

I have on this occasion reviewed my own studies of transfused Group O blood into recipients with blood of an agglutinable type, of which I have over 80 cases, with few, unfortunately, studied to the extinction of the transfusion. I have endeavored to draw some conclusions from these data by extending the slope of the curve of elimination to a base line. This is admittedly not accurate in the individual case, but appears to give interesting average results. In 8 cases, uncomplicated by malignancy, in which postoperative transfusions were given, the average time taken for unagglutinable cells to come to base was 124 days. In 16 cases of pernicious anemia it was 110 days. In addition there were in these groups cases which



showed no drop in the count while under observation and therefore no estimation could be made. On the other hand 10 cases with malignancy averaged 52 days. Seven cases of jaundice averaged 46 days. In a case of aplastic anemia with smallpox studied for 26 days the curve of the count of transfused corpuscles would have come to base in 41 days, while a case of splenic anemia in an infant by this method showed elimination of a first transfusion in 44 days and of a second in 55 days. In a total of 24 cases, in which hyperthyroidism and severe chronic infection are included, the average of the apparent length of life of the transfused blood was 52 days. But in 5 instances in which death was imminent the life of the transfused cell was even shorter.

It would seem, then, that we will have to regard the erythrocyte not as an entity, but as an integral part of the organism. Castle and Minot<sup>57</sup> in their article on the anemias in Oxford Medicine introduce Boycott's conception of the "erythron," which is understood to be the circulating blood and the organ from which it arises. I believe, however, that before the picture is complete we will have to consider a broader angle and include the effect of the endocrine system. As we pointed out in the beginning of this review, the red cell, both the compatible transfused cell and that produced in the body, probably is capable of undergoing repair, and it is by virtue of its capacity to repair itself that it survives the buffeting of the circulation. In a body in which the anabolic processes as compared with the catabolic processes are on the down grade, as in the terminal stages of disease, or in the presence of malignancy, one would expect the red cell to suffer with other body tissues and its life in the blood stream to be shortened.

The new knowledge on the interplay between the cortical and anterior pituitary hormones and their control of the up-build and destruction of protein, and their relation to insulin in carbohydrate metabolism as illustrated by the work of the Cori's on the enzyme complex hexokinase, may be intimately related to the problem of the life of the red cell. This question has already begun to interest biochemists. In the 1946 meeting of the Federation of American Societies for Experimental Biology a study was reported by Gonzales and Angerer<sup>58</sup> on the effect of adrenalectomy on the respiration of the erythrocytes of rats. They found a 41 per cent decrease in oxygen consumption in a Krebs-Ringer suspension of washed rat red cells five days after adrenalectomy and noted that the decrease was perceptible within forty-eight hours.

If we may assume that the life of the red cell in the rat is longer than five days, which would seem to be a safe assumption, this finding would indicate that the erythrocyte is dependent upon the adrenal for something that maintains its capacity to utilize oxygen and brings it into relationship with the organism in its ability to maintain itself in the circulation.

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# STUDIES ON FREE ERYTHROCYTE PROTOPORPHYRIN, PLASMA IRON AND PLASMA COPPER IN NORMAL AND ANEMIC SUBJECTS\*

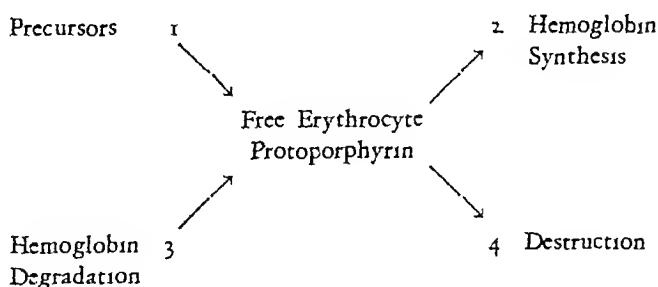
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THE VARIOUS anemias have been studied and classified clinically, morphologically, therapeutically and to a less extent etiologically, but comparatively few chemical studies have been made. Investigation of the chemical changes accompanying the various types of anemia offers a new approach to their study and gives rise to the hope that as the specific defects are elucidated their correction will become simplified. The purpose of this paper is to present data on free erythrocyte protoporphyrin, plasma iron, and plasma copper in normal subjects and in subjects with various types of anemia.

## REVIEW OF LITERATURE

The presence of protoporphyrin free in erythrocytes in addition to that in the hemoglobin molecule was reported by van den Bergh and co-workers<sup>1</sup> in 1928 and since then has been confirmed repeatedly.<sup>2-7</sup> Grotepass<sup>8</sup> and subsequently Watson, Grinstein and Hawkinson<sup>6</sup> demonstrated that this protoporphyrin is identical with the protoporphyrin of hemoglobin, namely, protoporphyrin 9, type III. A logical assumption is that this protoporphyrin is an intermediate compound in the synthesis of hemoglobin and that the free material is found because it has not been utilized for hemoglobin synthesis.

The amount of free protoporphyrin in the erythrocyte may be postulated as depending upon the relative rates of (1) synthesis of protoporphyrin from precursors, (2) utilization of the formed protoporphyrin for hemoglobin synthesis, (3) formation of protoporphyrin from hemoglobin in the intact erythrocyte, if such occurs, and (4) destruction of protoporphyrin, if this takes place. This may be represented diagrammatically.



Data have been presented elsewhere<sup>10</sup> which indicate that reticulocytes contain more free erythrocyte protoporphyrin than do mature red corpuscles. It has been reported that the protoporphyrin content of the bone marrow is increased when the percentage of normoblasts is increased.<sup>11</sup> In experimental hemolytic anemias the erythrocyte protoporphyrin did not increase when blood destruction was maximal but rose, instead, during the regenerative phase when reticulocytosis was marked.<sup>10</sup> It has been suggested that increased EP usually signifies uncompleted hemoglobin synthesis which may be the consequence

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of the liberation of immature cells or is due to iron deficiency or to factors interfering with the utilization of iron in the synthesis of hemoglobin is, for example, lead poisoning.<sup>5, 10</sup>

The manner in which protoporphyrin is broken down is unknown. It has not been demonstrated that destruction can take place in the intact red cell without the porphyrin being first formed into hemoglobin. The intravenous injection of protoporphyrin is not followed by a significant increase in the excretion of either bilirubin or coproporphyrin in the dog.<sup>12</sup> There is evidence, however, to indicate that under certain circumstances increased free erythrocyte protoporphyrin may result from degradation of hemoglobin in intact erythrocytes. It has been demonstrated that following sterile incubation of red cells for twenty-four to forty-eight hours there is an increase in the erythrocyte protoporphyrin.<sup>5</sup> Furthermore, red cells taken from the splenic vein following splenic stasis were found to have a greater protoporphyrin content than the cells in the splenic artery.<sup>10</sup> Although the demonstration that protoporphyrin is formed from hemoglobin in intact erythrocytes *in vitro* does not necessarily indicate that such a mechanism exists *in vivo*, there is considerable evidence that hemoglobin degradation can take place within the red cell *in vivo*. It would seem, however, that this takes place through a bile-pigment-iron native globin complex similar to or identical with the pseudohemoglobin of Barkan or the verdohemoglobin of Lemberg<sup>6</sup> rather than by liberating protoporphyrin.

The literature dealing with plasma iron and with iron metabolism in general was reviewed recently by one of us (GEC).<sup>15</sup> Plasma iron has been the subject of many investigations. Early work was unsatisfactory because of lack of reliable methods. Several satisfactory methods are now available.<sup>15, 16, 17</sup> It seems clear that plasma iron functions as transport iron. The amount of iron in the plasma is affected by the rate of absorption of iron, the balance between that going to and from the tissues, and the equilibrium between the amount used for hemoglobin formation and that coming from hemoglobin catabolism. Recent studies have shown that the iron in plasma is bound by a specific  $\beta_1$  globulin (Fraction IV-7 of Cohn).<sup>16, 17</sup>

The copper content of plasma in anemic states has been studied very little. Until recently, progress in this field of investigation was retarded by the lack of a simple reliable method. The normal serum copper content has been reported by several investigators to be approximately 70 to 160  $\mu\text{g}$  per cent.<sup>18-21</sup> Elevated serum copper values have been reported in pregnancy<sup>19, 20</sup> and in infections accompanied by anemia.<sup>19, 21, 22</sup> Plasma copper values in other clinical states accompanied by anemia have not been reported except by Locke, Main and Rosbach<sup>23</sup> and it has been shown that the method which they use is unreliable.<sup>18</sup>

Keilin and Mann<sup>24</sup> isolated a copper-protein compound of unknown function from serum as well as from red cells to which they gave the name of haemocuprein. The significance of this compound is unknown.

## METHODS

The hematologic methods used in this study have been described elsewhere.<sup>2</sup> Hemoglobin was determined by the photoelectric oxyhemoglobin method, using an Evelyn photoelectric colorimeter standardized by the Van Slyke procedure. Erythrocyte protoporphyrin determinations were made by the method of Grinstein and Watson.<sup>25</sup> The plasma iron was measured according to the procedure of Kitzes, Elvehjem and Schutte<sup>13</sup> or by the method of Barkan and Walker.<sup>14</sup> Both methods gave excellent recoveries (95 to 103 per cent of added iron). The method of Cartwright, Jones and Wintrobe<sup>18</sup> was followed for the determination of plasma copper. This method has been shown to be accurate within  $\pm 10$  per cent.

Values for erythrocyte protoporphyrin (EP) are expressed throughout the paper in  $\mu\text{g}$  per 100 ml of red blood cells. Plasma iron (PI) and copper (PCu) are expressed in  $\mu\text{g}$  per 100 ml of plasma. Ht refers to volume of packed red cells in ml/100 ml. MCV refers to mean corpuscular volume in  $\mu\text{m}^3$ . MCH refers to mean corpuscular hemoglobin in  $\mu\text{g}$ . MCHC refers to mean corpuscular hemoglobin concentration in per cent.

Each of the three chemical determinations, EP, PI and PCu, were done several or sometimes many times on each patient. The values expressed in the tables are either representative or are means for the given case.

### RESULTS

*A Normal values* The values for free erythrocyte protoporphyrin (EP), plasma iron (PI) and plasma copper (PCu) determined in normal adults are presented in tables 1 and 2. The subjects studied were not usually examined completely but they were apparently healthy. Although the ages of the 68 men and 66 women varied from 20 to 63 years, there was a disproportionate number of young individuals,

TABLE 1—*Normal Subjects Erythrocyte Protoporphyrin ( $\mu\text{g}/100\text{ ml RBC}$ )*

	Males	Females	Total
Observations	33	33	66
Range	13-79	16-140	13-140
Mean	32	39	35
Geometric Mean	28	34	31
Geometric Mean $\pm 2\text{ SD}$	11-70	14-83	12-79

TABLE 2—*Normal Subjects Plasma Iron and Plasma Copper ( $\mu\text{g per cent}$ )*

	Plasma Iron			Plasma Copper		
	Males	Females	Total	Males	Females	Total
Observations	49	43	92	52	53	105
Range	43-210	28-202	28-210	86-161	87-161	86-161
Mean	105.1 $\pm 4.3$	104.3 $\pm 5.5$	104.7 $\pm 3.4$	114.4 $\pm 2.2$	122.7 $\pm 1.5$	118.6 $\pm 1.2$
Standard Deviation	$\pm 30.3$	$\pm 36.4$	$\pm 32.8$	$\pm 15.5$	$\pm 10.6$	$\pm 12.5$
Mean $\pm 2\text{ SD}$	45-166	32-177	39-170	83-145	102-144	94-144

approximately two-thirds of each group being between 20 and 30 years of age. However, the data do not suggest that there is any variation related to age. In many of the individuals examined, several determinations were made of one or more of the three chemical constituents studied. The figures analyzed in tables 1 and 2 represent a single value for each person, usually the first obtained in the year 1947.

A total of fifty-six EP determinations in 33 males and fifty-two determinations in 33 females has been made. A frequency distribution curve of the determined values of erythrocyte protoporphyrin reveals a marked skew to the right (fig. 1). By using the logarithm of each value a curve more nearly approaching the normal was obtained. The analysis in table 1 was therefore carried out by this method. In males the observed values ranged from 13 to 70  $\mu\text{g}$  per 100 ml of red blood cells.

but only three values were above  $57 \mu\text{g}$ . In females the range was 16 to  $140 \mu\text{g}$  but only one value was below  $20 \mu\text{g}$  and only two were above  $68 \mu\text{g}$ . The difference between the geometric means for the two sexes is suggestive but not highly significant since the difference is only 1.7 times the standard error of the difference. Slight skew is still present after attempted normalization by use of logarithms and it is our conclusion that the standard deviation given in table 1 is too large. From inspection of the frequency distribution curve we have arbitrarily selected 13 to  $55 \mu\text{g}$  per 100 ml of RBC as the normal range in males and 16 to  $70 \mu\text{g}$  as the normal range in females. No attempt has been made to determine in detail the variation

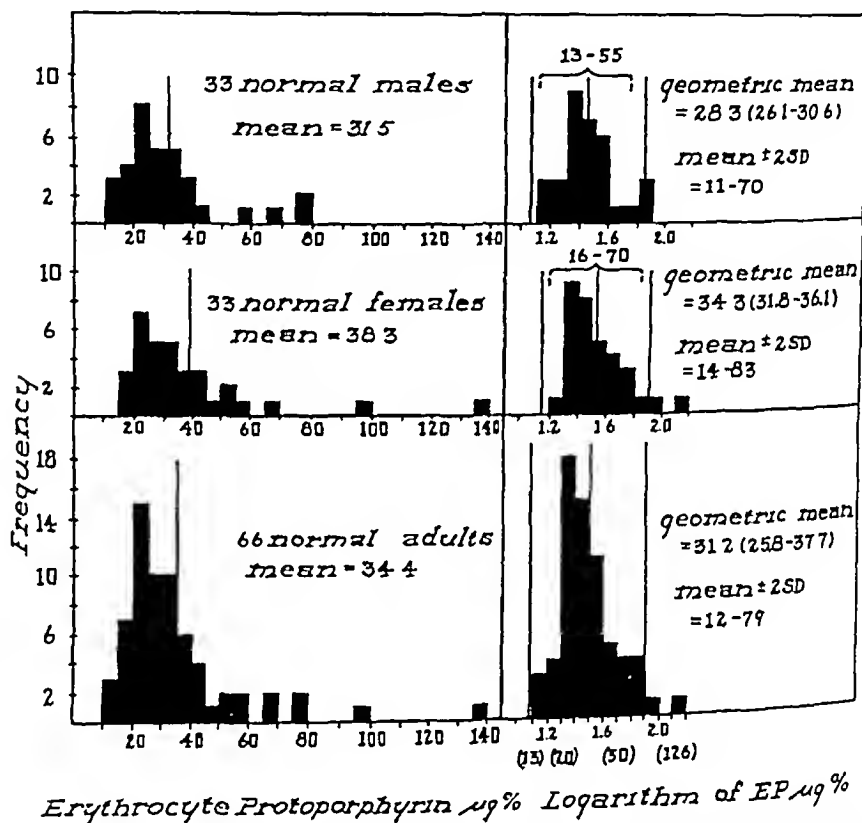


FIG. 1. Distribution of normal values for erythrocyte protoporphyrin. The graphs at the right are plotted in logarithmic values. The vertical lines represent the mean,  $+2$  S.D., and  $-2$  S.D.

in EP occurring over a period of time in individuals. The variation was usually not marked. The variation in females was not related to the menstrual cycle. Simultaneous reticulocyte counts were not done.

A total of ninety-four determinations of plasma iron on 49 normal males and one hundred and two determinations on 43 normal females has been made. The frequency distribution approximates a normal curve in each sex. The means for the two sexes are almost identical and the standard deviations are of the same order. As can be seen from table 2, the variation in normal values is great. This seems to be a reflection of the variation in each individual. In each of 3 males and 6 females from six to twenty-one determinations were made over a period of six months to two years. The means for these 9 individuals ranged from 68 to  $140 \mu\text{g}$  per cent

However, the variation in each individual was of the same order as that between the individuals. Thus, the average coefficient of variation in these 9 individuals was 40 per cent while that of the entire group was 31 per cent. Because of the close approximation of the distribution of plasma iron values to the normal curve the mean value  $\pm 2$  standard deviations has been used to indicate the normal. No relationship was noted between the plasma iron levels in females and the menstrual cycle. In one individual a series of determinations was made during the course of the day. In this instance there was no definite trend, the values from one morning to the next varying more than throughout the day. No postprandial change was noted in this individual.

TABLE 3—*Pernicious Anemia*

Name	Age	Sex	RBC	Hgb	Ht	MCV	MCH	MCHC	Retics	E P	P I	P Cu
			Million c mm	Gm %	cc %	c $\mu$	$\gamma\gamma$	%	%	$\mu\text{g}$ %	$\mu\text{g}$ %	$\mu\text{g}$ %
M B	74	F	1.75	6.4	18.5	106	37	35	0.4	30	222	100
A B	44	F	4.42	12.2	40.5	92	28	30	0.2	34	175	
R T	79	F	2.68	10.3	31.5	112	38	33	1.6	36	73	109
R Y	78	F	2.95	9.7	35.0	119	33	28	0.4	50	89	
S H	73	F	2.84	11.3	33.0	116	40	34	0.2	28	41	149
D M	46	F	2.35	9.5	26.5	113	40	36	1.9	41	149	115
H M	72	F	1.28	4.6	16.0	125	36	29	2.0	17	155	
L P	77	F	3.15	11.5	34.5	110	36	33	0.4	33	48	137
A S	82	F	2.70	9.7	28.5	105	36	34	0.2	26	113	128
M S	80	F	1.45	5.9	18.0	124	41	33	3.2	17	167	
T T	64	M	1.43	6.5	18.5	130	45	35	0.1	34	111	86
S S	69	M	1.50	5.9	17.8	119	39	33	3.2	9	282	
A G	74	M	1.34	4.9	16.0	120	36	30	1.8	44	250	183
C S	61	M	0.92	4.1	12.0	130	45	34	0.8	37	114	185
J W	60	M	1.31	4.6	15.0	116	35	31	1.2	—	222	—
N F	79	F	1.54	5.4	18.0	117	35	30	3.0	—	252	—
L R	26	F	1.54	4.5	17.5	113	29	26	1.5	—	28	—
J S	54	M	2.11	9.2	26.8	127	43	36	0.2	34	300	135
A B	69	M	1.38	5.1	14.4	104	37	35	2.0	29	201	108
N B	67	M	1.99	8.0	23.4	118	40	34	1.0	36	129	140

A total of seventy-five determinations of plasma copper on 52 normal males and seventy-five determinations on 53 females has been made. The frequency distribution curve in males approximates a normal curve but in females, though the range is the same as in males, most of the values are grouped close to the mean. The mean for the females is significantly higher than that for males. The variation was not great, the coefficient of variation being 13.6 per cent for males, 8.6 per cent for females, and 9.5 per cent for the group as a whole. The coefficient of variation in 4 males and 5 females in each of whom five determinations were made was 5.4 to 16.1 per cent with an average of 10.9 per cent. The mean value  $\pm 2$  standard deviations has been used to indicate the limits of normal.

**B Pernicious Anemia** The results obtained in 20 patients with pernicious anemia in relapse are shown in table 3. Erythrocyte protoporphyrin determinations were



made on 17 patients. In 16 of these the values were within the normal range and in one patient (S S) the values were low repeatedly. The mean for the entire group was  $31 \mu\text{g}$  per 100 ml of red blood cells as compared with the normal of  $35 \mu\text{g}$  (table 1). One patient (R T) is of particular interest. This patient was admitted to the hospital on November 15, 1945, in relapse at which time the EP was  $36 \mu\text{g}$ . She responded well to folic acid therapy but failed to return and received no further therapy. She was readmitted in relapse one year later with fever and sub

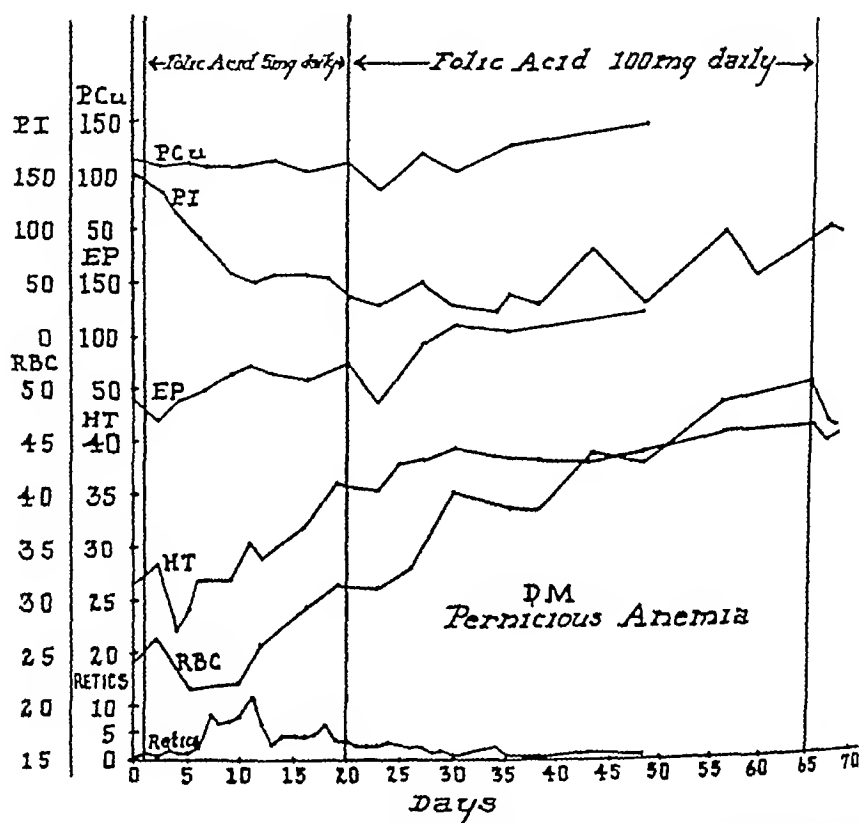


FIG. 2. Pernicious anemia in relapse (case D M) treated with folic acid orally. During therapy hypoferrremia developed and there was a gradual rise in EP to above normal. There was no significant change in plasma copper.

PCu refers to plasma copper expressed in  $\mu\text{g}$  per cent, PI to plasma iron expressed in  $\mu\text{g}$  per cent, EP, free erythrocyte protoporphyrin expressed in  $\mu\text{g}$  per 100 ml of red cells, Ht, volume of packed red cells in ml per 100 ml, RBC, red cell count in millions per c mm. The reticulocytes are expressed as per cent of red cells.

acute bacterial endocarditis. On the second admission the volume of packed red cells was 19 ml per 100 ml, the mean corpuscular volume  $110 \mu\text{m}^3$  and the EP  $290 \mu\text{g}$  although the plasma iron was  $268 \mu\text{g}$  per cent. Thus, it seems, patients with pernicious anemia in relapse are, at least in the presence of infection, capable of synthesizing excess protoporphyrin. In those patients who showed a satisfactory response to liver extract or folic acid therapy, there was a marked rise in EP above normal during the reticulocytosis. As the reticulocytosis subsided there was a fall in EP. This is not demonstrated in figures 2, 3 and 4 since the patients were given suboptimal doses of folic acid and a marked reticulocytosis did not occur. A sharp

rise in EP associated with reticulocytosis has been observed in other patients treated with optimal doses of liver or folic acid <sup>10</sup> As demonstrated in figures 2, 3, and 4, as the anemia was relieved, there was a gradual rise in EP to the upper limits of normal. In table 4, values for EP are presented for patients in relapse and remission. In remission the EP tended to be in the upper limits of normal or even above normal. It is of interest that EP in remission was always higher than during relapse.

Plasma iron determinations have been made on 28 patients with pernicious anemia in relapse. The data on 20 of these patients are presented in table 3. Of the

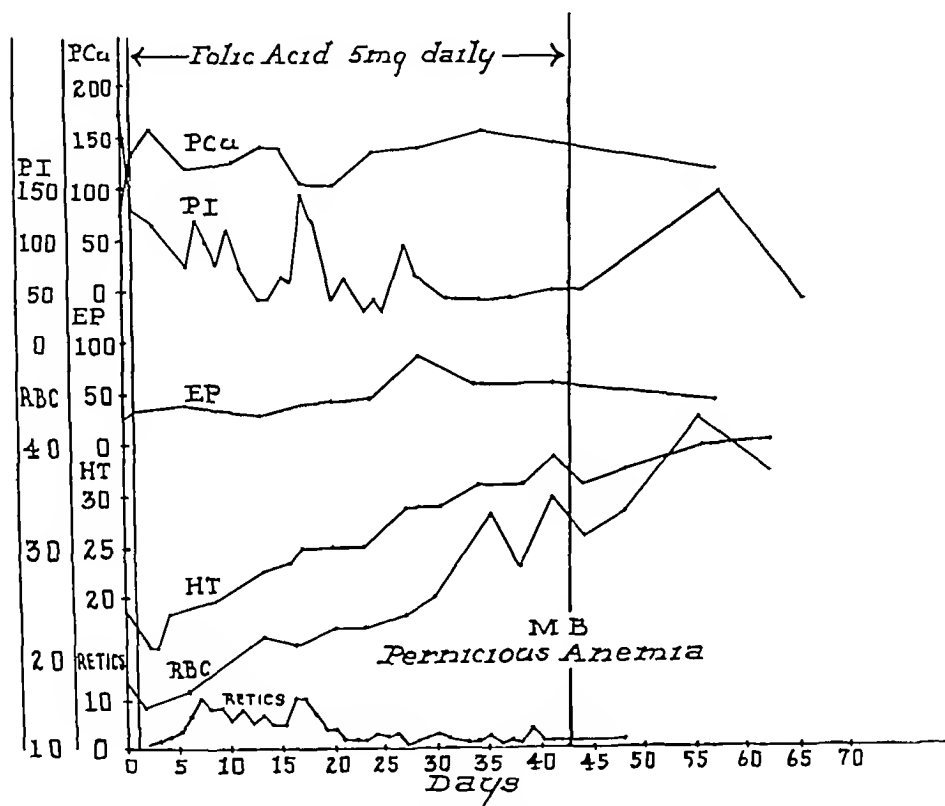


FIG 3 Pernicious anemia in relapse (case M B) treated with folic acid orally, 5 mg per day. During therapy slight hypoferrremia developed and there was a rise in erythrocyte protoporphyrin. There was no significant change in the plasma copper. For symbols see figure 2.

28 patients, in 16 (57 per cent) the values were within the normal range, in 11 (39 per cent) there was an elevation of the plasma iron, and in one patient the value was below the normal. Whatever the initial plasma iron may have been, hypoferrremia developed during the stage of rapid blood regeneration following therapy. This is illustrated in figures 2 and 3. In many the hypoferrremia persisted into the remission (table 4).

Plasma copper determinations have been made in 12 patients with pernicious anemia in relapse (table 3). The mean for the entire group was 137  $\mu$ g per cent. In 9 patients the values were within the normal limits and in 3 hypercupremia was noted. During therapy and in remission (table 3) there was no consistent trend in

either direction although in the one patient with an initial low-normal value there was a significant rise from 86  $\mu\text{g}$  to 125  $\mu\text{g}$

**C Iron Deficiency** The results obtained in 13 patients with varying degrees of anemia due to iron deficiency are presented in table 5. Erythrocyte protoporphyrin determinations were made on all 13 and in each case the value was found to be above the normal. The mean for the group was 207  $\mu\text{g}$  per 100 ml of red blood cells. No correlation between the degree of microcytosis or the degree of hypochromia and the magnitude of EP increase was noted. There was a rough correlation between the duration of the anemia and the level of EP. During the first

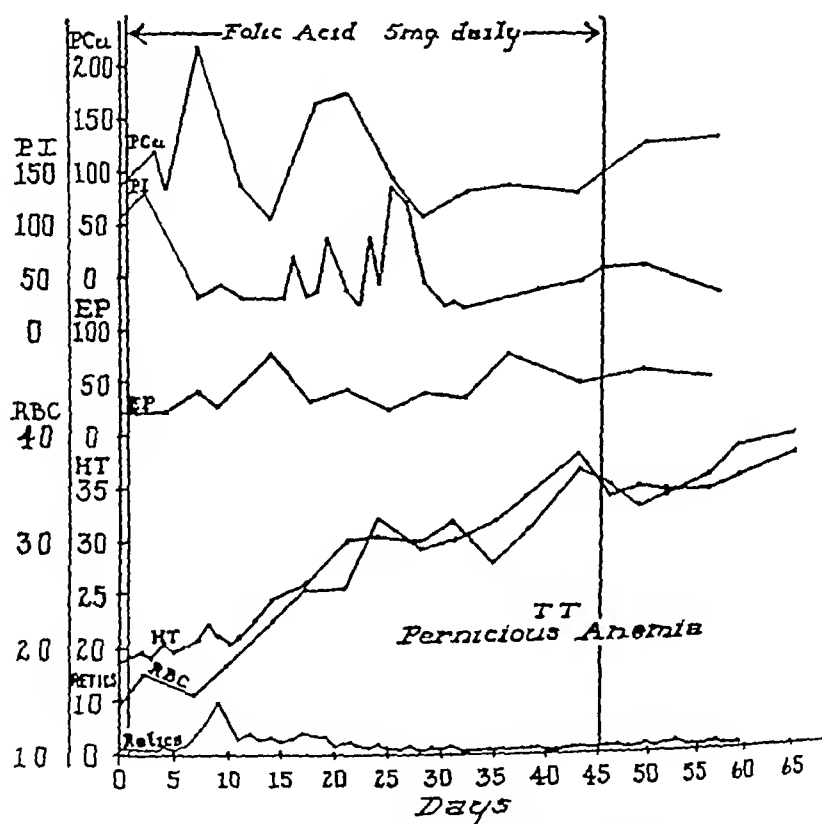


FIG. 4. Pernicious anemia in relapse (case T T) treated with folic acid orally, 5 mg per day. During therapy a slight hypoferrremia developed and there was a gradual increase in protoporphyrin. There were rather large fluctuations in plasma copper. For symbols see figure 2.

10 to 20 days following the onset of iron therapy, that is, during the period of reticulocytosis, there was a slight increase or no change at all in the EP. Then as the volume of packed red cells, mean corpuscular volume and mean corpuscular hemoglobin concentration returned to normal the EP diminished. This reached normal only some time after the blood had returned to normal (figs 5, 6, 7).

Plasma iron determinations were made in 13 patients. The results are presented in table 5 and are uniformly low. The mean for the entire group is 23  $\mu\text{g}$  per cent as compared with the normal of 105  $\mu\text{g}$ . During therapy (figs 5, 6, 7) there was a slow rise in plasma iron but not until some time after the blood had returned to normal did the plasma iron reach normal.

TABLE 4—*Pernicious Anemia before and after Therapy*

Patient	Period	Ht.	E.P	P I	P Cu
		cc. %	μg %	μg %	μg %
M B	Relapse	19	30	222	100
	Remission	34	60	108	131
S H	Relapse	33	28	41	149
	Remission	40	73	37	148
D M	Relapse	26	41	41	115
	Remission	42	116	45	140
L P	Relapse	35	33	48	137
	Remission	38	44	31	—
A S	Relapse	28	26	113	128
	Remission	39	36	60	143
M S	Relapse	18	17	167	—
	Remission	44	52	62	—
T T	Relapse	18	34	111	86
	Remission	40	78	23	125
S S	Relapse	18	9	282	—
	Remission	41	57	111	—
A G	Relapse	16	44	250	183
	Remission	43	88	36	—

TABLE 5—*Iron Deficiency*

Name	Age	Sex	RBC	Hgb	Ht	MCV	MCH	MCHC	Retic	E P	P I	P Cu
			Million c mm	Gm %	cc %	c μ	γγ	%	%	μg %	μg %	μg %
E Mc	66	F	3 47	5 4	21 0	60	16	26	1 0	475	16	
H R	23	M	3 95	5 8	23 2	59	15	25	—	389	15	
G A	60	M	3 13	4 8	21 0	67	15	23	4 0	183	25	177
V C	50	F	5 50	8 4	34 0	62	15	25	0 2	99	30	128
J H	33	F	4 70	9 7	35 0	74	21	28	0 5	100	31	
J H	71	F	5 01	7 8	29 5	59	16	26	1 7	216	20	210
G J	73	M	3 52	7 2	32 5	92	20	22	1 4	166	21	
H W	65	F	4 84	7 7	29 0	59	16	26	4 0	319	15	130
J J	49	F	4 88	9 0	35 0	72	18	26	3 0	100	19	145
D L	58	M	3 05	5 9	22 0	72	19	27	1 8	145	11	205
G L	45	F	4 38	7 2	31 0	70	16	23	1 0	100	32	
D H	49	M	4 47	8 7	32 6	73	20	27	1 6	200	27	155
J H	20	F	4 24	9 3	32 6	77	22	28	0 6	184	31	183

Plasma copper determinations were made in 8 patients (table 5). In 6 the values were found to be high and in 2 the values were within normal limits. No cor-

relation could be found between the degree of anemia and the degree of hypercupremia. Plasma copper was followed during therapy in one patient (fig 7). In this patient there was a fall in the copper which began about twenty days after the onset of therapy and reached normal at about the time the blood returned to normal and before the plasma iron or EP had reached normal.

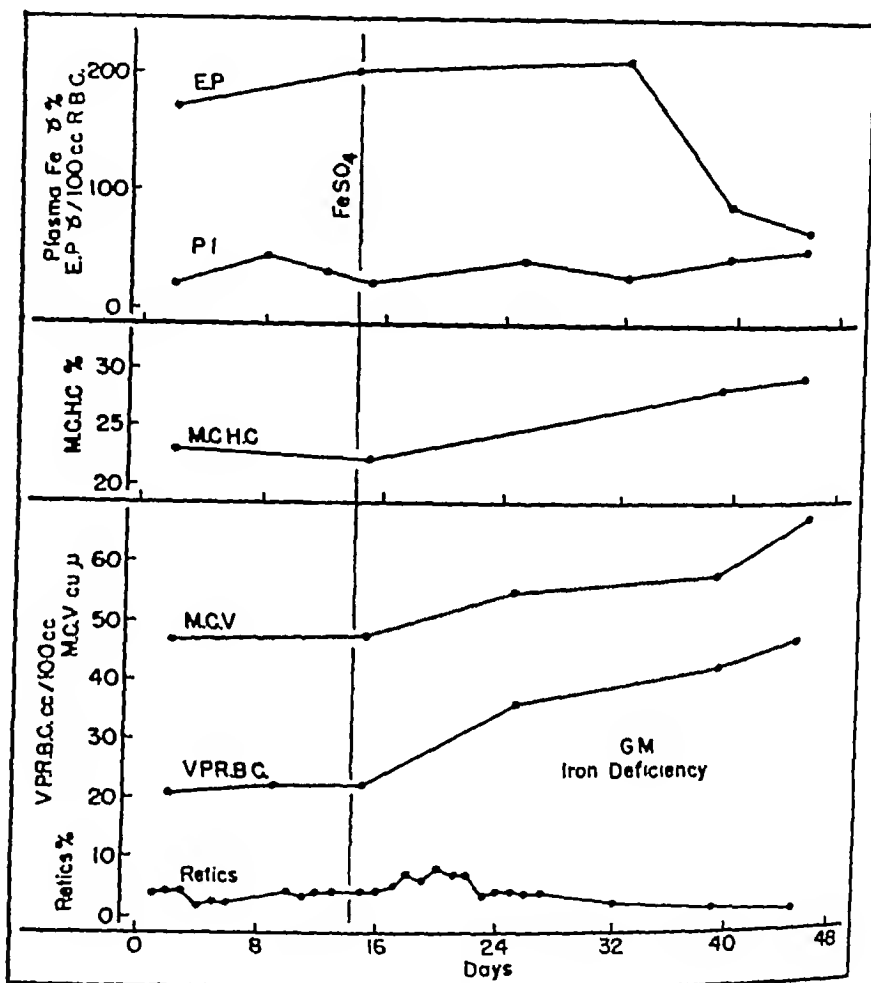


FIG 5 Iron deficiency anemia (case G M) with hypoferremia and an increase in EP. Treatment with ferrous sulfate was followed by a significant, though delayed, fall in EP and a gradual increase in plasma iron as the anemia was relieved. VPRBC refers to volume of packed red cells in ml per 100 ml (Ht), MCV, to mean corpuscular volume in  $\mu$ , MCHC, mean corpuscular hemoglobin concentration in per cent. For other symbols see figure 2.

**D Anemia of Infection** As previously reported, the anemia associated with chronic infection is accompanied by an elevated erythrocyte protoporphyrin, low plasma iron and high plasma copper.<sup>22</sup> The results in 10 patients not previously recorded are presented in table 6. These data confirm our previous findings. The erythrocyte protoporphyrin was elevated in 9 of the 10 patients, hypoferremia and hypercupremia were present in all those so examined. A number of additional patients with acute and subacute infections have been studied and data are now available concerning the rapidity and sequence of the changes in relation to the onset of the disease and during convalescence.

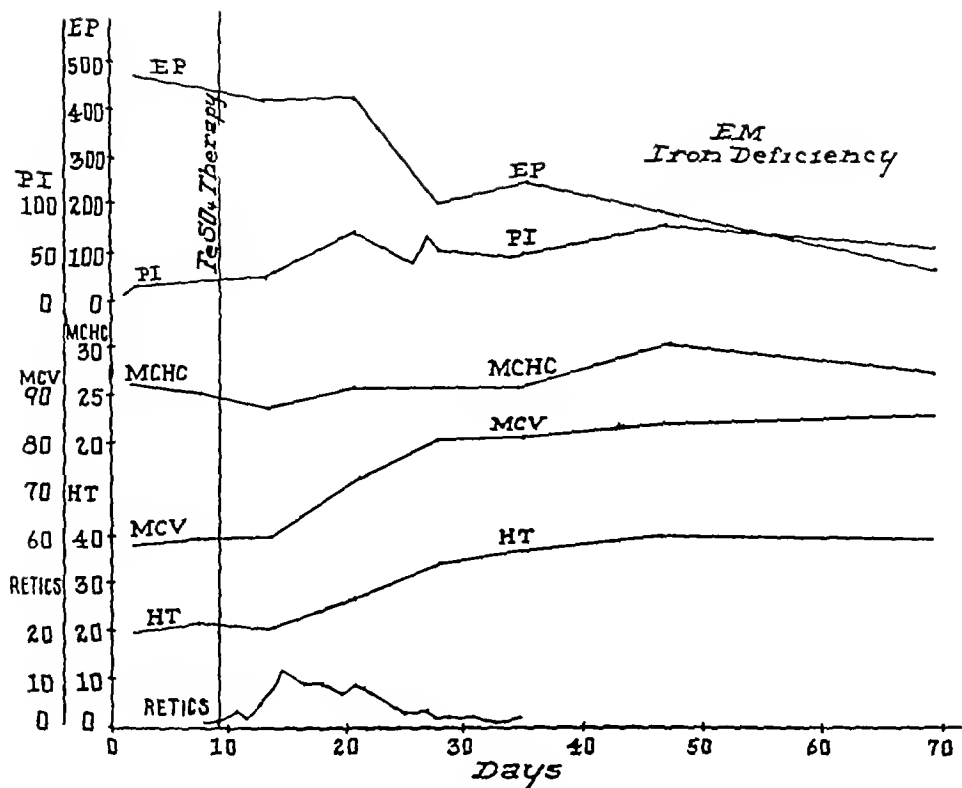


FIG 6 Iron deficiency anemia (case E M) with hypoferrremia and an increase in EP. During therapy with ferrous sulfate there was a significant decrease in EP and a gradual rise in plasma iron. The volume of packed red cells and indices reached normal before the EP returned completely to normal. For symbols see figures 2 and 5.

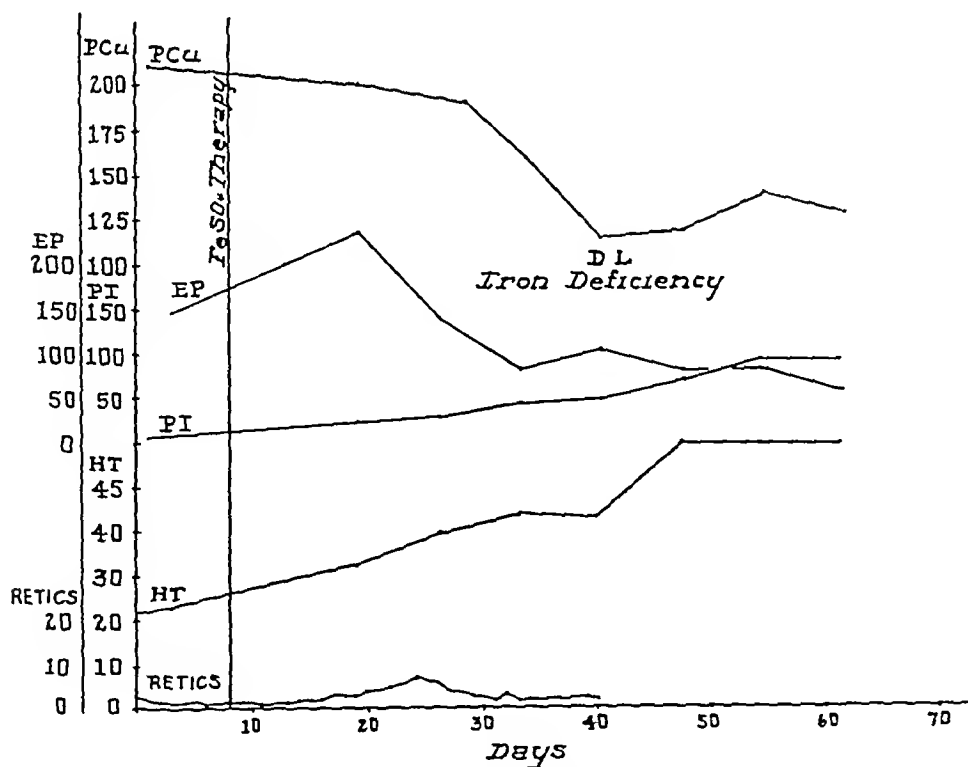


FIG 7 Iron deficiency anemia (case D L) with hypoferrremia, hypercupremia and increase in EP. During therapy with ferrous sulfate a marked drop in plasma copper took place. There was an initial increase in EP followed by a drop to normal. The plasma iron rose gradually. For symbols see figure 2.

In acute infections with fever such as uncomplicated lobar pneumonia, bacterial meningitis, pharyngitis, scarlet fever and otitis media, hypoferremia was found to develop within a few days after the onset of fever. This is illustrated in 2 patients with meningococcal meningitis (fig 8). When the disease was of short duration the plasma iron rose when the fever subsided and no anemia developed. Hypoferremia appears to be the first change to take place and occurs in a variety of conditions associated with fever. In figure 9 the effect of induced fever (typhoid vaccine) on the plasma iron is demonstrated. With each paroxysm the iron dropped markedly and returned rapidly to normal shortly after the cessation of fever. No anemia developed and there was no significant change in plasma copper or rise in erythrocyte protoporphyrin.

TABLE 6—*Chronic Infections*

Name	Age	Sex	Disease	Duration months	RBC million c mm	Hgb Gm %	Ht cc %	MCV cu	MCH γγ	MCHC %	Fe μg %	P I μg %	P Cu μg %
J W	28	M	Subacute Bacterial Endocarditis	9	3 82	8 9	28 5	75	23	31	266	25	171
D S	35	F	Subacute Bacterial Endocarditis	9	3 35	7 7	29 8	89	23	26	137	—	158
T M	78	M	Disseminated Tubercu- losis	12	—	10 0	31 5			31	128	36	159
R M	53	M	Lung Abscess	8	3 25	7 0	27 0	83	22	26	564	30	167
J L	63	M	Osteomyelitis	24	3 82	9 0	30 2	79	24	30	221	17	—
A B	54	M	Pneumonia	1	4 26	10 5	33 0	78	25	32	91	15	169
T E	45	M	Actinomycosis		4 34	11 8	39 0	90	25	28	63	14	179
A R	45	M	Lung Abscess	1	3 36	10 5	32 2	96	34	36	345	27	199
J K	69	F	Empyema	3	3 76	11 4	35 2	93	30	32	100	20	189
I C	36	F	Empyema	1	4 28	11 7	35 0	82	27	33	55	30	214

The effect on the plasma iron of a more prolonged illness is demonstrated in figures 10 and 11. In patient W V (fig 10) there was an initial hemolytic phase with slight jaundice accompanying lobar pneumonia. The total serum bilirubin was 1.5 mg per cent. With this there was an initial hyperferremia which was followed by the hypoferremia usually associated with infection. This persisted for about twenty-five days without anemia developing. Patient I C (fig 11), a woman 36 years of age who had pneumonia and an acute lung abscess, was seen on about the twenty-first day of illness. The plasma iron at this time was 30 μg per cent and the volume of packed red cells 35 ml. The disease responded rapidly to parenteral penicillin therapy and the plasma iron rose promptly. Two patients (F L, pyo-nephrosis and D S, empyema) with more chronic illnesses are described in figures 12 and 13. Significant anemia was present in both. The plasma iron did not reach normal in either patient until after the anemia disappeared.

In acute infections with hypoferrremia no change in EP has been noted. This is illustrated in figures 8, 9 and 11. Only when the infection persisted for a month or more or when significant anemia developed did a rise in EP occur. This rise sometimes reached its maximum after the maximal development of anemia. This is illustrated in figure 14. Return of EP to normal took place slowly (figs 10, 12, 13 and 14) and did not reach normal until long after the anemia had disappeared.

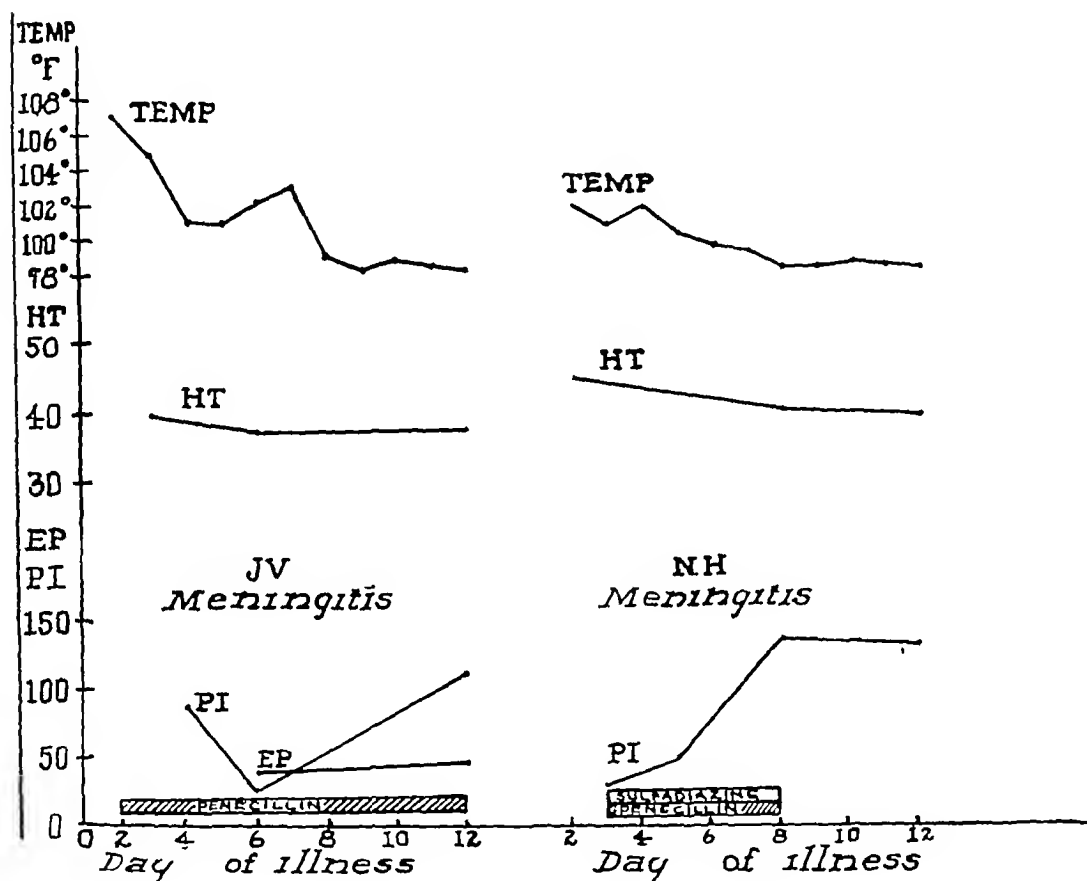


FIG 8 The effect of acute infections on the plasma iron as exemplified in two patients with meningococcal meningitis (J V and N H). Hypoferrremia developed early in both patients. The iron returned rapidly to normal as the fever subsided. No anemia developed and there was no increase in EP. For symbols see figure 2.

The rise in EP seemed to be correlated with the duration of the infection and to a less extent with the severity of the anemia.

The rise in plasma copper took place some time after the development of hypoferrremia but was observed before or in the absence of a rise in EP (fig 11). As can be seen in figure 9 no significant change took place in the plasma copper during the paroxysms of fever. In chronic infections with anemia (table 6) hypercupremia was almost invariably present. The copper returned to normal more rapidly than did the EP or iron and became normal about the time of the disappearance of anemia, as illustrated in figure 10.



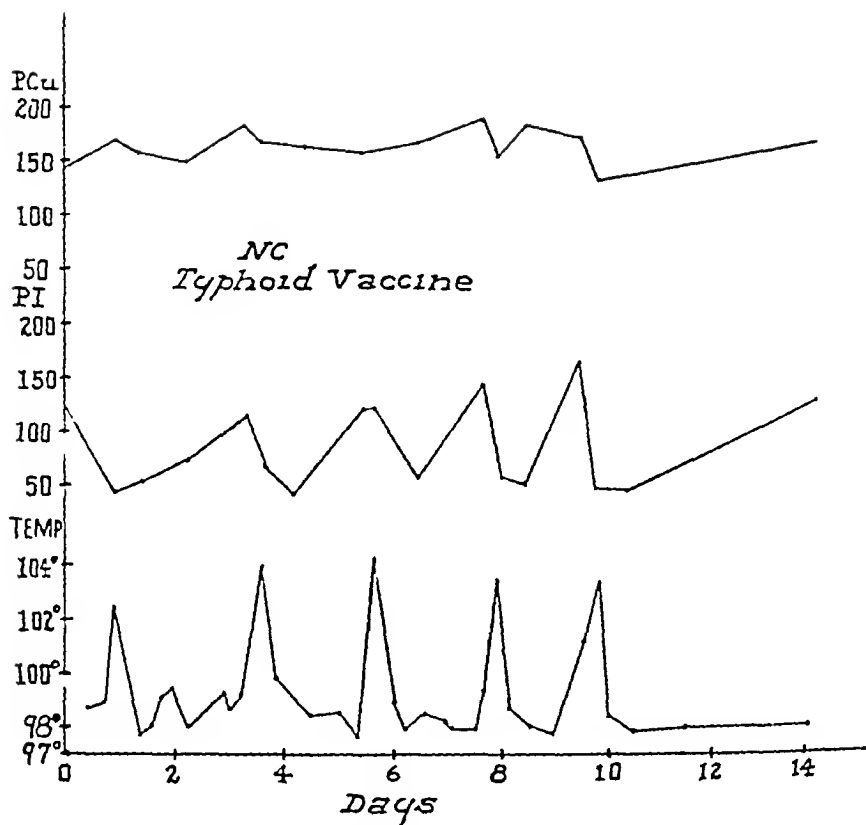


FIG 9 The effect of induced fever (typhoid vaccine) on the plasma iron. With each paroxysm the plasma iron dropped markedly and returned to normal shortly after the cessation of fever. There was no significant change in the plasma copper. For symbols see figure 2.

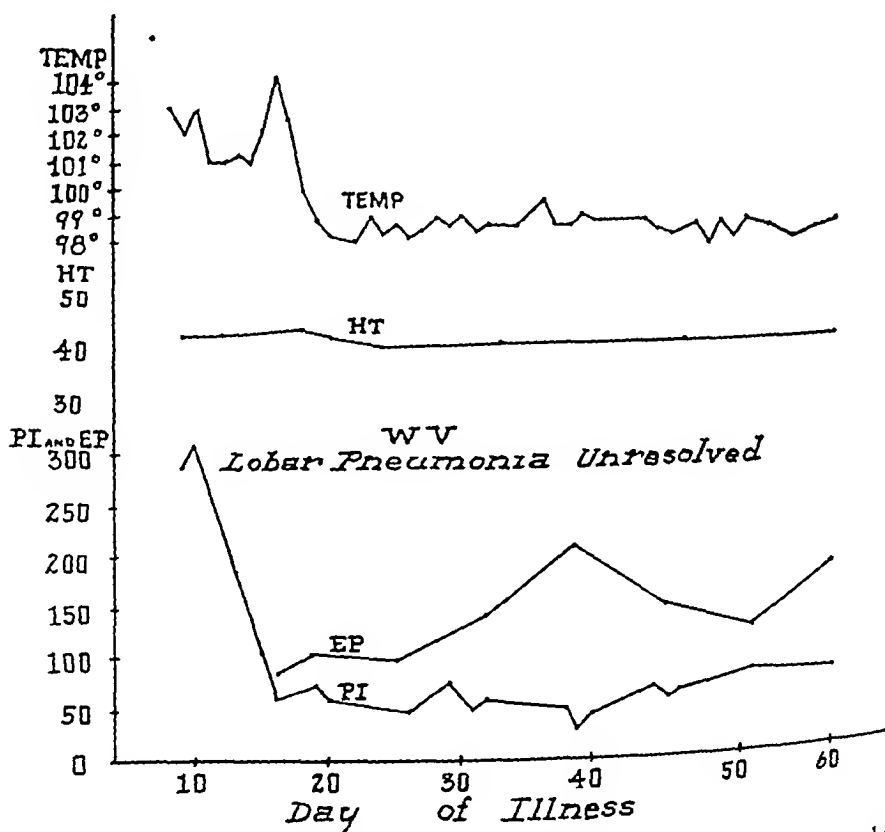


FIG 10 Unresolved lobar pneumonia (case W V). There was an initial hemolytic phase with jaundice, bilirubinemia and hyperferremia. Following this hypoferremia developed and a rise in EP occurred. For symbols see figure 2.

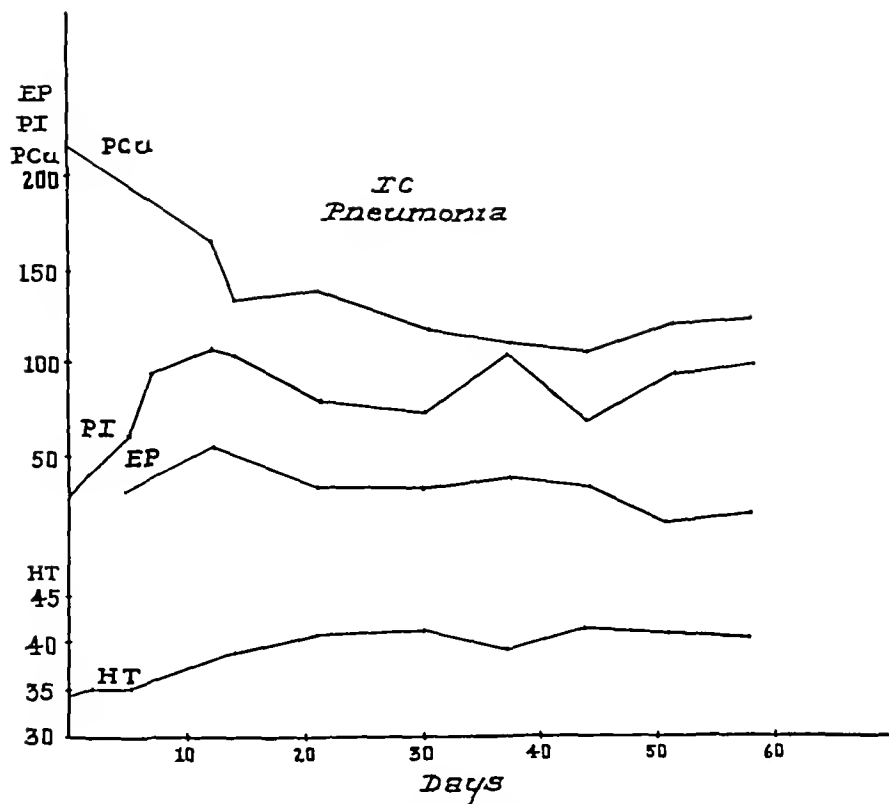


FIG. 11. A patient with lobar pneumonia and early lung abscess (case I C) with anemia, hypoferrremia and hypercupremia which responded well to parenteral penicillin. As the anemia disappeared the plasma iron rose and plasma copper returned to normal. There was no significant change in EP. For symbols see figure 2.

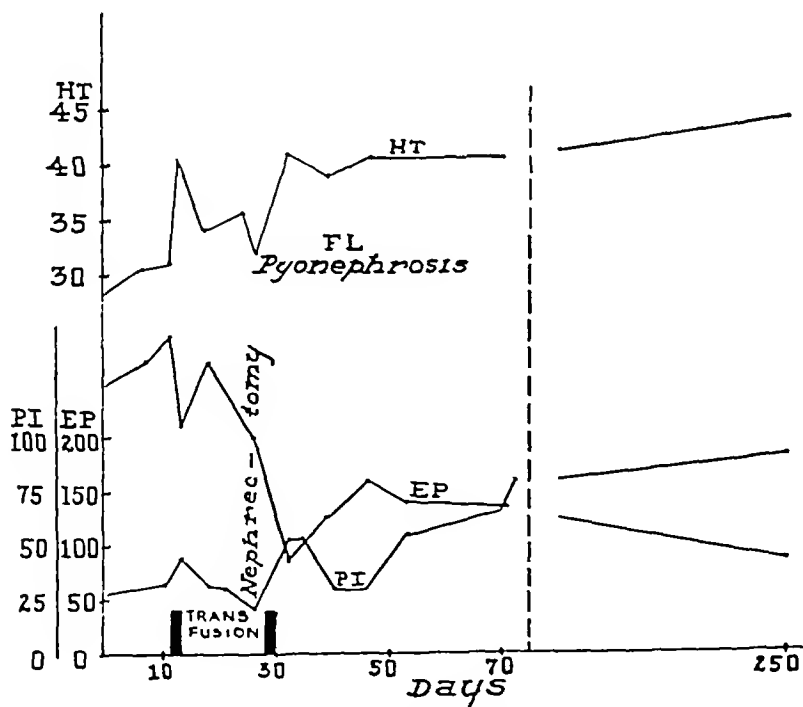


FIG. 12. Chronic infection (pyonephrosis, case F L) with anemia, hypoferrremia and an increase in EP. Kidney function was not diminished. Following nephrectomy the anemia disappeared, the plasma iron rose, and the EP decreased. For symbols see figure 2.

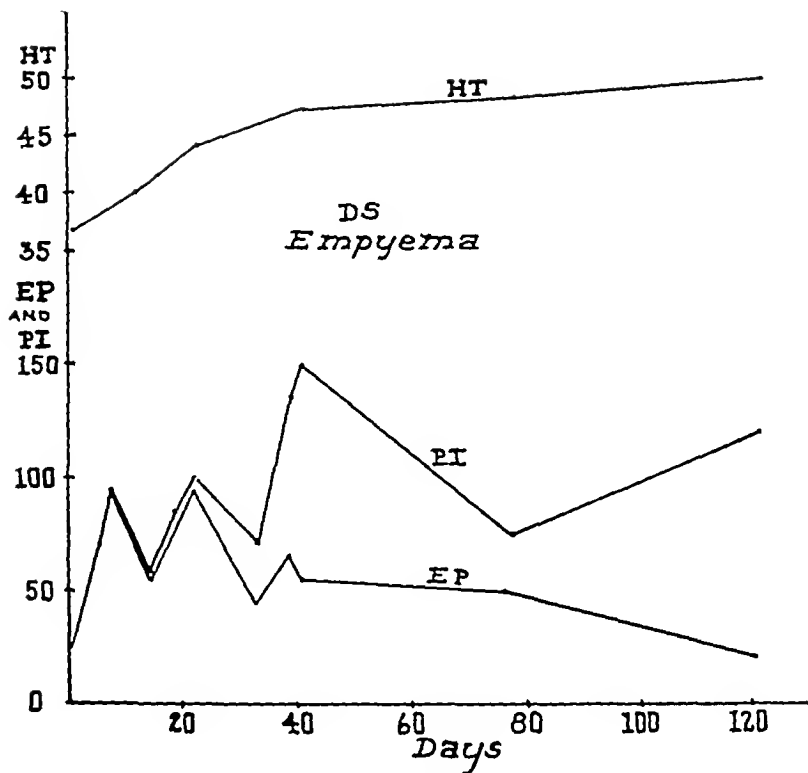


FIG 13 Chronic infection (empyema, case D S) with anemia, hypoferremia and an increase in EP. As the infection subsided the EP diminished to normal and the plasma iron rose. For symbols see figure 2.

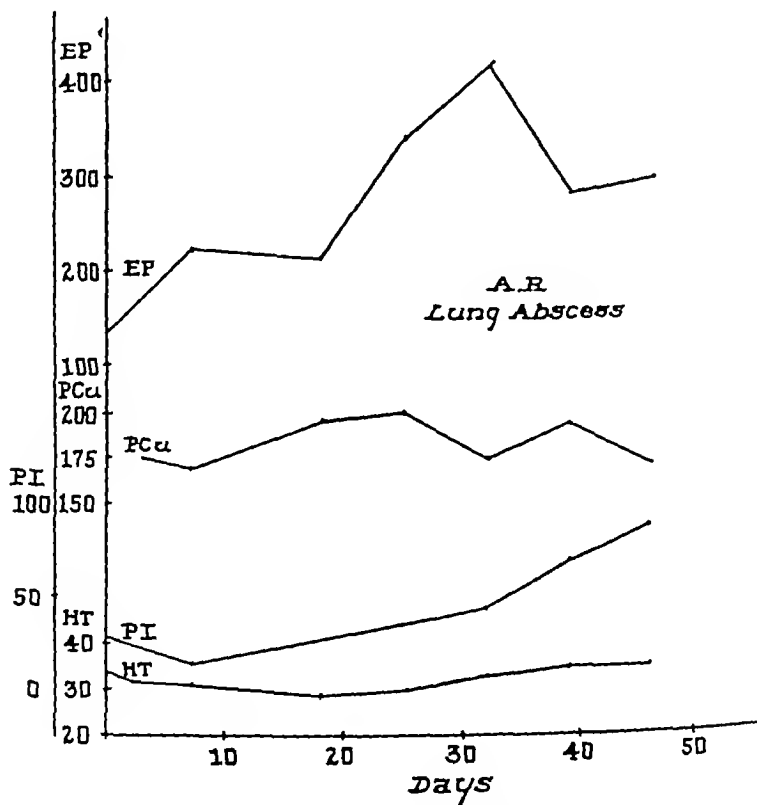


FIG 14 Chronic infection (lung abscess, case A R) with anemia, hypoferremia, hypercupremia and an increase in EP. The hypoferremia and hypercupremia developed early. The maximal rise in EP occurred later at the time when the anemia was most severe. This patient was unusual in that the plasma iron rose in spite of persistent anemia. For symbols see figure 2.

It is interesting that one patient with anemia and chronic osteomyelitis developed severe nephritis during the course of his illness. The serum albumin fell to 1.73 grams per cent and the serum globulin to 1.79 grams per cent. At the same time the plasma copper dropped from 246  $\mu$ g per cent to 40  $\mu$ g per cent and remained at this low level.

*E Anemia of Nephritis* The results in 10 patients with anemia associated with various types of nephritis are presented in table 7. In 8 of the 10 patients the erythrocyte protoporphyrin was elevated and in 2 the values were normal. In 4 of the patients the plasma iron was low and in 6 normal. Plasma copper determinations were made on 5 patients. In 4 the values were high and in the remaining patient the value was normal. Sufficient study has not been made to correlate

TABLE 7—Nephritis

Name	Age	Sex	Type	RBC	Hgb	Ht	MCV	MCH	MCHC	Re tic	EP	PI	P Cu	BUN	TP	Alb	Glob
				mil lion c mm	Gm per- cent	cc %	c. $\mu$	$\gamma\gamma$	%	%	$\mu$ g %	$\mu$ g %	$\mu$ g %	mg %	Gm %	Gm %	Gm %
A B	73	F	Chronic Arterio- sclerotic	2.84	8.1	24.0	85	29	34	4.8	138	32	240	51	6.5	4.2	2.3
D H	18	M	Chronic Glomeru- lonephritis	3.05	7.5	29.0	95	24	24	—	80	21	136	27	4.8	2.5	2.3
R C	69	M	Embolic Suppura- tive	2.72	7.2	26.5	75	26	35	2.8	92	21	—	35	5.6	2.9	2.7
G C	50	M	Malignant Nephro- sclerosis	3.50	9.5	31.1	90	27	31	—	71	35	193	91	7.3	4.6	2.7
C C	34	M	Chronic Glomeru- lonephritis	3.32	10.0	30.2	91	30	33	1.2	178	76	—	75	—	—	—
J A	60	M	Chronic Glomeru- lonephritis	2.33	7.2	20.5	88	31	35	—	101	111	165	154	5.8	4.0	1.8
A M	39	F	Chronic Glomeru- lonephritis	2.25	7.0	20.5	91	31	34	—	59	117	183	66	7.6	4.2	3.4
I M	37	F	Chronic Pyelone- phritis	3.44	9.5	32.0	93	28	30	1.8	18	81	—	21	5.1	2.7	2.4
C J	73	M	Nephrosclerosis	2.15	5.5	18.0	84	26	31	1.4	198	15	—	51	4.7	2.4	2.3
A J	63	M	Nephrosclerosis	5.40	15.0	48.5	90	28	31	—	100	115	—	20	—	—	—

these chemical changes with the type of nephritis nor with the other metabolic changes which take place in this disease.

*F Lymph node disorders and Leukemia* Observations in 19 patients with various types of lymph node disorders and leukemia are presented in table 8. All determinations were made prior to treatment with x-ray, nitrogen mustard or urethane. Erythrocyte protoporphyrin determinations were made on 19 patients. In 12 patients the values were elevated and in 7 normal. Plasma iron determinations were made on 19 patients. In 12 patients the values were normal, in 6 low, in 1 elevated. Determinations of plasma copper were made in 6 patients and values greater than normal were found in all. The rise in EP was more marked in the 'lymphoma' group than in the leukemia group. Hypoferremia was not observed in the leukemia group.

*G Thalassemia* Observations in 6 patients with thalassemia minor and 5 patients with thalassemia major are presented in table 9. Unfortunately, EP determinations

## CHEMICAL CHANGES IN NORMAL AND ANEMIC SUBJECTS

could not be carried out except in one patient In all 6 of the patients with the minor variant of the disease the serum iron was normal In 4 of the 6, hyper-

TABLE 8 — *Lymph Node Disorders and Leukemia*

Name	Age	Sex	Disease	RBC Million c mm	Hgb Gm %	Ht cc %	MCV c $\mu$	MCH $\gamma\gamma$	MCHC c %	FP $\mu\text{g}\%$	PI $\mu\text{g}\%$	PCu $\mu\text{g}\%$	Comment
C B	25	M	Hodgkin s Disease	4 25	10 6	34 0	80	25	31	121	21	—	—
R F	57	M	Hodgkin s Disease	3 23	8 9	29 2	90	28	30	47	40	—	—
E G		F	Hodgkin s Disease	5 39	14 2	43 4	80	26	33	182	52	—	—
F H	40	M	Hodgkin s Disease	3 52	8 6	25 0	71	24	34	61	31	—	Retics 7 4%
M P	27	F	Hodgkin s Disease	2 42	5 1	20 0	83	21	26	322	36	—	—
W R	57	M	Hodgkin s Disease	3 12	6 8	25 0	80	22	27	200	56	—	—
A S	34	M	Hodgkin s Disease	2 89	8 4	30 5	105	29	30	79	72	199	—
N S	26	F	Hodgkin s Disease	3 10	5 3	20 0	65	17	26	115	70	—	—
H S	21	F	Hodgkin s Disease	4 50	11 5	38 5	85	28	30	58	29	212	—
M T	36	F	Hodgkin s Disease	3 69	11 3	32 2	87	31	35	94	34	—	—
C W	24	M	Hodgkin s Disease	4 25	9 8	32 5	77	23	30	98	16	222	—
P K	56	M	Reticulum Cell Sarcoma	4 25	9 7	34 0	80	23	29	236	23	165	—
M G	36	F	Reticulum Cell Sarcoma	5 24	11 6	37 8	71	22	31	51	15	—	WBC 465,000
F M	33	F	Chronic Myelocytic L	2 40	6 0	18 0	75	25	33	114	94	—	WBC 160,000
M M	58	F	Chronic Myelocytic L	4 00	12 0	36 0	90	30	33	40	46	—	WBC 24,750
C C	79	F	Chronic Myelocytic L	4 67	12 7	40 5	87	27	31	48	45	173	WBC 151,000
G W	56	F	Chronic Myelocytic L	3 84	9 5	28 5	74	25	33	65	60	174	WBC 37,000
H R	56	M	Chronic Lymphocytic L	1 61	4 7	13 0	81	29	36	61	292	—	WBC 38,000
G C	25	M	Acute Lymphoblastic L	4 95	14 6	43 0	87	29	34	26	133	—	—

TABLE 9 — *Thalassemia*

Name	Age	Sex	Type	RBC Million c mm	Hgb Gm %	Ht cc %	MCV c $\mu$	MCH $\gamma\gamma$	MCHC %	Retics %	PI $\mu\text{g}\%$	PCu $\mu\text{g}\%$
J T *	45	F	Minor	6 34	11 8	36 5	58	19	32	1 4	103	146
A A	28	F	Minor	5 52	10 5	34 0	61	19	31		144	111
K Z	46	F	Minor	5 25	11 7	35 5	68	22	33		76	138
M D	41	F	Minor	5 47	12 9	37 2	63	21	34		90	226
M C	65	F	Minor	5 92	12 6	37 0	63	21	34		176	218
J T	31	F	Minor	6 05	11 9	35 0	58	20	34	4 9	110	153
P F	9	M	Major	3 15	7 8	23 0	73	25	34	19 0	202	181
V P †	1	M	Major	3 35	5 8	26 0	77	17	22	5 0	80	330
S F	2	F	Major	2 15	4 2	14 0	65	20	30	2 2	211	161
A P	7	M	Major	2 09	4 3	14 2	68	21	30	3 2	310	—
N P	7	M	Major	1 97	4 0	13 2	67	20	30		288	—

\*, EP 65  $\mu\text{g}$

†, Infection (T 101°)

cupremia was present In thalassemia major hyperferremia was present in 4 of the 5 patients In the fifth an infection with fever was present and the iron was within

the normal limits. In the 3 patients with the major form of the disease, in whom serum copper determinations were made, hypercupremia was present in each.

In figure 15 the negative effects of intravenously administered pyridoxine on the hyperferremia of two patients with thalassemia major are presented. These same 2 patients had been previously reported as benefiting from combined prolan B and pyridoxine therapy.<sup>27</sup>

H *Miscellaneous conditions* Observations in a variety of hematologic conditions other than those discussed above are presented in table 10. Several findings are

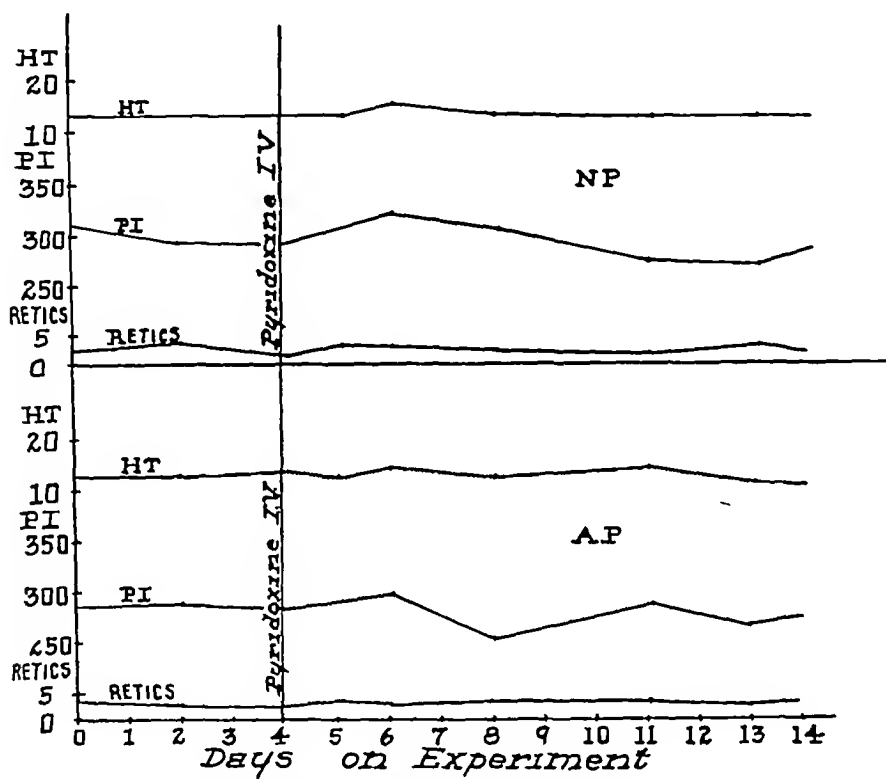


FIG 15 Two patients (N P and A P) with thalassemia major and hyperferremia. The hyperferremia was not affected by intravenous administration of 50 mg pyridoxine hydrochloride daily. For symbols see figure 2.

worthy of comment. In the 4 patients with aplastic anemia who were studied the plasma iron was markedly elevated in each. Erythrocyte protoporphyrin determinations were made in 2. In one patient the value was slightly elevated and in the other normal. Two patients with myelophthisic anemia were found to have high EP. A patient with hemochromatosis had a normal plasma iron and a low plasma copper. In 2 patients with plumbism there was a marked elevation of the EP. Sufficient data are not available on hemolytic anemia to draw conclusions since only 2 patients were studied and the results differed.

#### DISCUSSION

Our values for the normal EP correspond with those which Watson, Grinstein and Hawkinson<sup>5</sup> obtained in 12 normal individuals, although the range in our

series is somewhat higher. The data for normal plasma iron are similar to those recorded by others<sup>15</sup> except that, whereas most observers have found that the mean value for females is significantly lower than for males, in our series the means were

TABLE 10 — *Miscellaneous Conditions*

Name	Age	Sex	Condition	RBC	Hgb	Ht	MCV	MCH	MCHC	Retics	EP	PI	PCu
				Million c mm	Gm %	cc %	cu	γγ	c°	c°	μ%°	μ%°	μ%°
D H	26	M	Aplastic Anemia <sup>1</sup>	2 50	9 8	28 0	112	39	35	0 1	46	318	169
P D	61	M	Aplastic Anemia <sup>2</sup>	1 96	5 4	16 5	84	28	33	0 1	70	260	120
L B	39	F	Aplastic Anemia <sup>3</sup>	2 54	7 5	22 0	87	30	37			226	
N G	35	F	Aplastic Anemia <sup>3</sup>	1 50	3 8	13 0	87	26	30	0 8		222	
E G	63	M	Myelophthisic Anemia <sup>4</sup>	1 69	3 4	13 5	80	28	35	3 4	143	175	
M G	67	F	Myeloideriosis <sup>5</sup>	2 77	9 5	31 8	111	34	30	9 6	138	143	
N A	15	M	Congenital Hemolytic Icterus <sup>2</sup>	3 29	11 5	34 6	105	35	33	4 8	45	35	
T A	27	F	Congenital Hemolytic Icterus <sup>2</sup>	3 02	11 0	29 0	96	36	38	11 2	149	88	115
V P	24	M	Plumbism	5 07	13 8	42 0	83	27	33	1 0	274	191	104
J D	25	M	Plumbism <sup>2</sup>	4 38	10 6	36 0	82	24	29	3 0	206	125	100
E P	25	M	Hemochromatosis	4 41	15 0	48 3	109	34	31		28	147	60
N P	68	M	Polycythemia	7 70	22 6	72 0	92	29	32		25	184	
J R	32	M	Polycythemia <sup>6</sup>	8 67	22 7	75 0	88	25	29		76	267	
J V	73	M	Polycythemia <sup>6</sup>	7 51	22 2	66 0	88	28	32	0 9	26	69	
F G	60	M	Polycythemia	7 25	17 7	60 0	83	25	30		22	125	
C D	70	F	Hypothyroidism <sup>7</sup>	4 10	12 0	33 0	80	27	36		52	77	111
A F	56	F	Hypothyroidism	4 33	11 3	36 0	83	26	31		80	75	
M S	55	F	Hypothyroidism	3 82	11 1	34 0	89	29	33		49	157	176
J O	61	M	Cirrhosis Liver	2 55	7 4	25 0	98	29	30	1 0	51	44	
T N	42	M	Cirrhosis Liver	3 48	10 4	32 0	92	32	33		80		
E P	19	F	Subacute Yellow Atrophy	4 27	12 5	39 0	91	29	32		40	108	122
L S	71	F	Banti's Syndrome <sup>2</sup>	3 68	12 5	32 0	87	34	39	1 4	35	99	
W G	57	M	Banti's Syndrome <sup>2</sup>	3 73	10 4	31 8	85	28	33	0 7	62	88	180
M P	72	F	Multiple Myeloma	3 68	11 1	35 0	95	30	32	0 1	46	46	
W W	46	M	Multiple Myeloma	3 81	10 8	32 5	85	28	33		53	69	135
C B	37	M	Constitutional Hyper- bilirubinemia	5 84	16 4	45 6	83	30	36	0 2	26	101	119

<sup>1</sup> Hypoplastic marrow, <sup>2</sup> Normoblastic marrow, <sup>3</sup> Fibrotic bone marrow, <sup>4</sup> Carcinoma prostate, Secondary, <sup>6</sup> Primary, <sup>7</sup> Diabetic

almost identical. The normal plasma copper values also agree with those obtained by others using reliable methods<sup>18-21</sup>

In table 11 the results of erythrocyte protoporphyrin, plasma iron and plasma copper studies in a variety of clinical conditions associated with anemia are summarized. In general, it was found that in pernicious anemia in relapse the EP was

normal, the plasma iron normal or high and the plasma copper usually normal. Anemia due to iron deficiency and chronic infections was accompanied by an elevated EP, hypoferremia and hypercupremia. In nephritis with anemia the EP was generally increased, the plasma iron was low or normal and the plasma copper was increased. Anemia associated with lymph node disorders or leukemia was accompanied by a normal or high EP, a low or normal plasma iron and an increase in plasma copper. In thalassemia minor the serum iron was normal, hypercupremia was found in 4 out of 6 patients. Thalassemia major was accompanied by both hyperferremia and hypercupremia. Hyperferremia was present in all 4 patients with aplastic anemia who were studied. In 2 patients with plumbism there was a marked increase in EP. Hypocupremia was encountered only twice, in one patient with severe nephritis and hypoalbuminemia and in one patient with hemochromatosis.

TABLE II—*Summary of the Data*

Condition	Erythrocyte Protoporphyrin				Plasma Iron				Plasma Copper			
	No Pts	Low	Normal	High	No Pts	Low	Normal	High	No Pts	Low	Normal	High
Pernicious Anemia	17	1	16	0	28	1	16	11	12	0	9	3
Iron Deficiency	13	0	0	13	13	13	0	0	8	0	2	6
Chronic Infections	10	0	1	9	9	9	0	0	9	0	0	9
Nephritis	10	0	2	8	10	4	6	0	5	0	1	4
Lymphoma or leukemia	19	0	7	12	19	7	11	1	6	0	0	6
Aplastic Anemia	2	0	1	1	4	0	0	4	2	0	1	1
Thalassemia Major					5	0	1	4	3	0	0	3
Thalassemia Minor	1	0	1	0	6	0	6	0	6	0	2	4
Hemochromatosis	1	0	1	0	1	0	1	0	1	1	0	0
Plumbism	2	0	0	2	2	0	1	1	2	0	2	0
Myelophthistic Anemia	2	0	0	2	2	0	1	1				
Hemolytic Anemia	2	0	1	1	2	1	1	0	1	0	1	0

In general it was found that in conditions characterized by hypoferremia, the EP and plasma copper were elevated (anemia of infection, iron deficiency, nephritis, lymph node disorders and leukemia). Analyzing the data in another way, it would seem that there was an increase in EP in anemic states associated with a normoblastic bone marrow due to a disturbance in hemoglobin synthesis, i.e., iron deficiency, anemia of infection, nephritis, lead poisoning and some cases of "lymphoma" and leukemia. In contrast, in pernicious anemia, which is characterized by a megaloblastic bone marrow, there was no increase in the protoporphyrin content of the erythrocytes. These observations are in accord with Stasney's direct observations on the protoporphyrin content of various types of bone marrow.<sup>11</sup> His studies suggested that normoblasts contain protoporphyrin in considerable amount, while megaloblasts do not.

A high EP has not been found, however, in all conditions associated with a normoblastic bone marrow. Thus, in the bone marrow of patients L. S. (Banti's syndrome), W. G. (Banti's syndrome) and N. A. (congenital hemolytic jaundice),



42, 32 and 40 per cent, respectively, of all of the nucleated cells were normoblasts. Yet the EP values in the blood were 35, 62 and 45  $\mu\text{g}$  respectively. Again, in pyridoxine deficiency anemia in swine<sup>13-29</sup> low values for EP have been found in the face of normoblastic marrow hyperplasia. Watson<sup>6</sup> determined the EP in a typical case of thalassemia major and found it to be only 20  $\mu\text{g}$  per 100 cc. He speculated that this might be because protoporphyrin is not formed in appreciable amounts in the early erythroblast stage. This could not be the explanation for the essentially normal EP values in our cases, cited above, since the normoblasts in the bone marrow were late forms. Further studies of the EP content of various types and stages of red blood cells are needed.

Theoretically it would seem that if hemoglobin synthesis is retarded due to factors other than a deficiency in protoporphyrin synthesis, the amount of free protoporphyrin might be increased since protoporphyrin would not be the limiting factor. This can be visualized as follows:

- (a) Porphyrin precursors  $\rightarrow$  Protoporphyrin
- (b) Protoporphyrin + Fe + Globin  $\rightarrow$  Hemoglobin

If reaction (b) does not proceed and reaction (a) continues, there would be an excess formation of protoporphyrin. By the same reasoning if reaction (a) is the limiting factor it might be expected that the EP would be low or normal.

As indicated earlier, there is evidence that reticulocytes contain more free protoporphyrin than mature cells.<sup>10</sup> It is not known whether this represents a small excess left over during hemoglobin synthesis or is merely a degradation product of hemoglobin. It would seem more logical to assume that the presence of free protoporphyrin in reticulocytes represents uncompleted hemoglobin synthesis. In the anemia of infection hemoglobin synthesis has been shown to be impaired.<sup>22</sup> An increase in EP under such circumstances could be readily understood. In iron deficiency anemia, lack of iron is the limiting factor. If there is no defect in protoporphyrin synthesis, one would expect an increase in EP. Any other conditions which impair hemoglobin synthesis without interfering with protoporphyrin production should be characterized by increased EP—unless protoporphyrin is broken down or removed at a rate sufficient to prevent accumulation. We have postulated that in pyridoxine deficiency protoporphyrin synthesis may be impaired<sup>29</sup> whereas in pernicious anemia, protoporphyrin accumulation may be prevented by its conversion to bilirubin,<sup>30</sup> thus explaining the low and normal EP values observed in these conditions, respectively, both of which are characterized by hyperferremia.

In the last analysis, however, one must concur with Watson who pointed out<sup>6</sup> that until it is determined whether the protoporphyrin of reticulocytes is merely a small excess left over during hemoglobin synthesis or is purely a degradation product of hemoglobin and until it is known whether or not protoporphyrin is eliminated or built up into additional hemoglobin in the circulating erythrocyte, its significance will remain uncertain.

The significance of the plasma iron is clearer than that of free erythrocyte protoporphyrin. Our observations are consistent with those described by

previous investigators, cited already. In conditions in which the amount of iron absorbed is decreased (inadequate dietary intake of iron, etc.) and in conditions in which the rate of elimination is increased (hemorrhage), the plasma iron is low. In conditions in which the amount of iron going to the tissues is increased (anemia of infection), or in conditions in which hemoglobin is being rapidly synthesized (e.g., pernicious anemia during treatment), the plasma iron is low. In conditions in which hemoglobin synthesis is reduced due to factors other than a lack of iron (pernicious anemia in relapse, thalassemia major, aplastic anemia, pyridoxine deficiency in swine), the plasma iron is high. In conditions in which hemoglobin catabolism is accelerated (hemolytic anemia) the plasma iron is high. More than one of these factors may be operating, as in hemolytic anemia where both hemoglobin synthesis and hemoglobin destruction are accelerated. In this event the plasma iron is dependent upon the balance of these factors and generally fluctuates, depending upon which factor predominates at a given time.

Knowledge of the absorption, function and metabolism of copper, especially in relation to erythropoiesis, is so limited that interpretation of our findings is difficult if not impossible. In general, plasma copper is a more stable constituent of blood than is the EP or plasma iron. An elevation of plasma copper has been a rather consistent finding in (1) the anemia of infection, (2) "lymphomas," (3) leukemia, (4) iron deficiency, (5) nephritis, and (6) thalassemia. No change was noted in pernicious anemia. Low plasma copper values were infrequent. We have observed hypocupremia only twice. In the patient with osteomyelitis and hypercupremia who subsequently developed nephritis, hypoalbuminemia and hypocupremia the last finding might be explained on the basis of the hypoalbuminemia if copper is bound to an albumin in the serum. The finding of hypocupremia in the patient with hemochromatosis is interesting in view of Mallory's theory<sup>28</sup> that hemochromatosis is due to copper poisoning. The hypocupremia might indicate rapid mobilization of the copper into the tissues.

#### SUMMARY

1. A total of 108 erythrocyte protoporphyrin determinations has been made in 66 normal individuals. The geometric mean  $\pm$  standard error of the mean was 31 (26-38).

2. A total of 196 determinations of plasma iron in 92 normal individuals was made. The mean  $\pm$  standard error of the mean was  $104.7 \pm 3.4$   $\mu$ g per cent.

3. In a total of 150 determinations of plasma copper in 105 normal individuals, the mean  $\pm$  standard error of the mean was  $118.6 \pm 1.2$   $\mu$ g per cent.

4. No significant difference in plasma iron was noted between the sexes but in females the plasma copper was significantly higher and the erythrocyte protoporphyrin slightly higher than in males.

5. Erythrocyte protoporphyrin, plasma iron, and plasma copper determinations have been made in over 112 patients with a variety of clinical conditions associated with anemia. In general, it was found that in pernicious anemia in relapse the erythrocyte protoporphyrin values were normal, the plasma iron normal or high and the plasma copper usually normal. Anemia due to iron deficiency as well as the

anemia of infection were accompanied by high values for erythrocyte protoporphyrin, hypoferremia and hypercupremia. In nephritis with anemia the erythrocyte protoporphyrin was generally increased, the plasma iron low or normal and the plasma copper increased. Anemia associated with lymph node disorders or leukemia was accompanied by a normal or high EP, a low or normal plasma iron and an increase in plasma copper. Thalassemia major was found to be accompanied by both hypercupremia and hyperferremia, in thalassemia minor the serum iron values were normal although hypercupremia was found. Hyperferremia was noted in aplastic anemia. In cases of plumbism the erythrocyte protoporphyrin was markedly increased. Hypocupremia was noted only twice, in one patient with severe nephritis and hypoalbuminemia and in one patient with hemochromatosis.

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## STUDIES IN IRON TRANSPORTATION AND METABOLISM

### VI ABSORPTION OF RADIOACTIVE IRON IN PATIENTS WITH FEVER AND WITH ANEMIAS OF VARIED ETIOLOGY\*

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RECENT studies of iron utilization have demonstrated that intravenously administered tracer doses of radioactive iron are utilized completely for hemoglobin synthesis by afebrile, iron-deficient patients or animals, and almost completely by normal subjects.<sup>1</sup> On the other hand, when hemoglobin synthesis is impaired, as in patients with refractory anemia or untreated pernicious anemia, in patients with febrile disorders, and in pigs with infection or pyridoxine deficiency, both the rate and completeness of utilization are decreased.<sup>1-3</sup> It was discovered, furthermore, that the amount of tagged iron which appears as hemoglobin in the peripheral blood of subjects with hemolytic anemia cannot be used as a measure of iron utilization because of the rapid rate at which isotopic hemoglobin is removed from the circulation. These results clearly indicate that the radioactive iron technic for studying iron absorption, as used in the past,<sup>4-12</sup> may give erroneously low values because only iron built into hemoglobin is measured, the assumption that this amount equals the quantity absorbed is not always justified. In the face of this new evidence, it is necessary to re-evaluate the ability of patients with impaired hemoglobin formation to absorb iron. Absorption of the metal by normal subjects should also be restudied because even though normal persons use tracer amounts of injected iron completely or almost completely for hemoglobin synthesis, absorbed iron goes into the portal rather than the systemic circulation and may, therefore, be handled in a different way. This report describes experiments designed to meet the above objections and to discover the physiologic pattern of iron absorption under a variety of pathologic influences.

The isotopic method for studying iron absorption has been extended to include not only measurement of the amount of iron converted into hemoglobin after an oral dose, but also determination of the unabsorbed portion which is eliminated in the feces. A standard dose of 1 mg of iron per kilogram of body weight has been selected. Any portion of the test dose not accounted for in the circulating hemoglobin and in the recovery from feces represents iron that has been absorbed but not immediately utilized. With this approach, the principle that iron-deficient subjects absorb larger amounts than do normal persons has been confirmed.<sup>4, 13, 14</sup> The amount retained by healthy men and women, however, has occasionally exceeded 10 per cent, because of the error inherent in the method, it was not possible to determine accurately how much might be stored without being used immediately for hemoglobin, but the quantity was not large. It was possible to demonstrate that patients with refractory anemia, untreated pernicious anemia,

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and fever often did absorb more iron than they were able to build into hemoglobin during the period of observation. One woman with Hodgkin's disease and hypochromic anemia, for instance, retained 50 per cent of the oral dose but used for hemoglobin synthesis only one-third of the amount absorbed.

These results are of considerable theoretic interest. It is now believed that the animal organism has an extremely limited capacity to excrete iron<sup>14, 15</sup> except by hemorrhage, and that the intestinal mucosa protects the body from accumulating toxic amounts of the metal by accepting or rejecting iron according to need.<sup>8, 16, 17</sup> The intestinal mucosa, according to this concept, is one of the major regulators of iron metabolism. Evidence indicating that patients with hypochromic anemia absorb more iron than do normal persons is compatible with this theory. However, the observation that patients with untreated pernicious anemia and refractory anemia absorb appreciable amounts of the metal in spite of the relatively large amounts of iron in their tissues, shows the regulatory effect of the intestinal mucosa to be less precise than was formerly believed. •

### MATERIAL AND METHODS

The subjects who volunteered for this study were healthy medical students, members of the laboratory staff, and patients on the Medical Service of the Barnes Hospital. The test dose of radioactive iron was given orally, at a level of 1 mg. of iron per kilogram of body weight, as ferrous chloride, reduced from the ferric state with ascorbic acid. The usual procedure was to give the test dose after a night's fast, but in a number of experiments the iron was given 3 to 5 hours after a meal. In one instance, through an error, the test dose was given immediately after the patient had eaten lunch and excellent absorption of the metal occurred (J. L., first dose, table 2, fig. 1). Quantitative fecal collections were made until radioactivity measurements showed that the 24 hour specimen contained less than 0.5 per cent of the activity in the test dose. In most of the subjects, fecal collections could be suspended after the sixth or seventh day. In two instances they were continued until the twelfth day. Since the method depended on getting complete stool collections, extreme vigilance was exercised by the laboratory staff. Only experiments in which there was satisfactory evidence of reliable collections have been included in the study. Blood was drawn at intervals of three days for determination of the radioactive iron in hemoglobin. The total amount of radioactivity in the peripheral blood was calculated by assuming the blood volume to be 80 cc. per kilogram of body weight. The error introduced by this assumption did not influence interpretation of results since the amount of iron found in the blood was usually small as compared with that recovered from the feces. Details of the technics for making the radioactivity determinations have already been published.<sup>18</sup>

The fresh fecal specimen was weighed and mixed thoroughly with water to which enough concentrated hydrochloric acid was added to bring the final concentration to approximately 0.6 N (50 cc. concentrated HCl per liter). The suspension was transferred quantitatively through a funnel to a liter volumetric flask, and enough water was added to adjust the volume to exactly one liter. The flask was stoppered and shaken vigorously for about three minutes. As quickly as possible, an aliquot sample (one twentieth to one tenth) of the stool suspension was measured into a Kjeldahl flask. The mixture was then digested with sulfuric and perchloric acids. The cooled digest was transferred to a volumetric flask, and an aliquot portion was taken for measurement of radioactivity. Five milligrams of inert iron were added as a carrier to the aliquot in a 40 cc. centrifuge tube and the iron was precipitated with NaOH, using phenol red as an indicator. The precipitate was thrown down by centrifugation, the supernatant liquid was discarded, and the precipitate was dissolved in 0.3 cc. of 3 M H<sub>2</sub>SO<sub>4</sub>. To this solution were added in the centrifuge tube about 10 cc. of a mixture of three parts of saturated ammonium oxalate solution and one part of saturated oxalic acid solution. The precipitated calcium and magnesium oxalate salts were thrown down by centrifugation and the supernatant solution was transferred quantitatively to the electroplating cell. The precipitate of oxalates was stirred with a few drops of 3 M H<sub>2</sub>SO<sub>4</sub> and

10 cc of the oxalate mixture, the tube was again centrifuged and the washings were combined with the material already in the electroplating cell. From this solution, the iron was electroplated onto a copper disk<sup>18</sup> and its radioactivity was measured with a Kip type Geiger counter tube.

Attention is directed to the fact that iron was not extracted from the fecal specimens by ether, as was done in certain earlier experiments.<sup>1</sup> Ether extraction was unnecessary because the radioactivity in these fecal specimens was great enough to permit high dilutions of the specimens. In these high dilutions, salts other than calcium and magnesium did not interfere with the determination.

TABLE 1—*Efficiency of Recovery of Radioiron Added to Feces*

Sample	Weight of Fresh Feces	Chemical Form of Radioiron Added	Method	Aliquot Counted	Radioiron Added	Radioiron Found	Recovery
	Gm				counts/min	counts/min	%
1	66	FeCl <sub>3</sub>	Ether extraction	1/6 75	642	599	93.5
2	72	FeCl <sub>3</sub>	Oxalate precipitation	1/50	64,320	64,320	100
3	72	FeCl <sub>3</sub>	Oxalate precipitation	1/100	128,600	131,600	102
4	72	FeCl <sub>3</sub>	Oxalate precipitation	1/100	257,200	233,680	91
5	50	Fe(OH) <sub>3</sub>	Oxalate precipitation	1/5000	3,600,000	3,396,000	94
6	50	Fe(OH) <sub>3</sub>	Oxalate precipitation	1/5000	3,600,000	3,490,000	97
7	50	Fe(OH) <sub>2</sub>	Oxalate precipitation	1/2500	1,440,000	1,352,000	94
8	50	Fe(OH) <sub>2</sub>	Oxalate precipitation	1/1000	720,000	668,000	93
9	50	FePO <sub>4</sub>	Oxalate precipitation	1/2000	1,440,000	1,451,000	100
10	50	FePO <sub>4</sub>	Oxalate precipitation	1/2000	1,440,000	1,374,400	95.5
11	40	FePO <sub>4</sub>	Oxalate precipitation	1/20	19,500	17,736	91
12	40	FePO <sub>4</sub>	Oxalate precipitation	1/100	97,500	98,200	101

TABLE 2—*The Absorption of Radioactive Iron by Patients with Hypochromic Anemia*

Patient	Date	Hematologic Data					Total Test Dose of Radioiron		Radioiron Recovered		
		RBC	Hb	Cell Vol	MCV	MCHC			Feces	Blood	Total
		millions	Gm	%	cu microns	%	mg/kg	counts/min	%	%	%
J L	2-24-45	4.24	9.1	32	76	2.8	1	4,440,000	38	57	95
J L	3-16-45*†	4.23	8.5	30	71	2.8	1	3,360,000	76	24	100
O J	10-7-46	3.81	5.4	22	58	2.4	1	1,280,000	64.5	36	100.5
M M	3-12-47	3.76	7.1	27	72	2.6	1	1,920,000	50	57	107
H N	5-3-47†	4.93	7.4	28	57	2.6	1	2,850,000	60	27	87
S W	8-12-47†	4.06	6.9	24	59	2.9	1	32,000,000	29	81	110

\* Forty-eight hours before the second test dose of radioiron, J. L. received 1320 mg Fe as colloidal Fe(OH)<sub>3</sub> intravenously.

† The blood volume of J. L. and of S. W. was measured by the Evans blue dye method.

‡ H. N. vomited four hours after taking the test dose of radioiron.

Prior to October, 1946, the radioactive isotope used was Fe<sup>59</sup>, prepared in the Washington University cyclotron. Since that time, we have used a mixture of Fe<sup>59</sup> and Fe<sup>55</sup> obtained from the Clinton Laboratories. On each day that determinations of radioactivity were made, the number of counts emitted by a standard prepared from the original iron solution was also determined several times; this value was used as the standard of reference for all calculations.

## RESULTS

### I. THE ACCURACY OF THE METHOD

Twelve recovery experiments were done to test the accuracy of the determination of radioactive iron in feces (table 1). In the first four, measured amounts of radio-

iron as ferric chloride were added to four different fecal suspensions which had been acidified with HCl. Because radioiron of weak activity was added to a large mass of feces in the first experiment, ether extraction according to the method previously described<sup>1</sup> was used, ether extraction was not done during any of the remaining determinations described in this report. In the next six experiments, the isotope in the form of ferric hydroxide, ferrous hydroxide, or ferric phosphate, was added to the fecal specimen, carefully mixed, and incubated at 37°C for twenty-four hours before the determination was made, in the last two experiments radioactive iron as ferric phosphate was added to the solid specimen and an excess

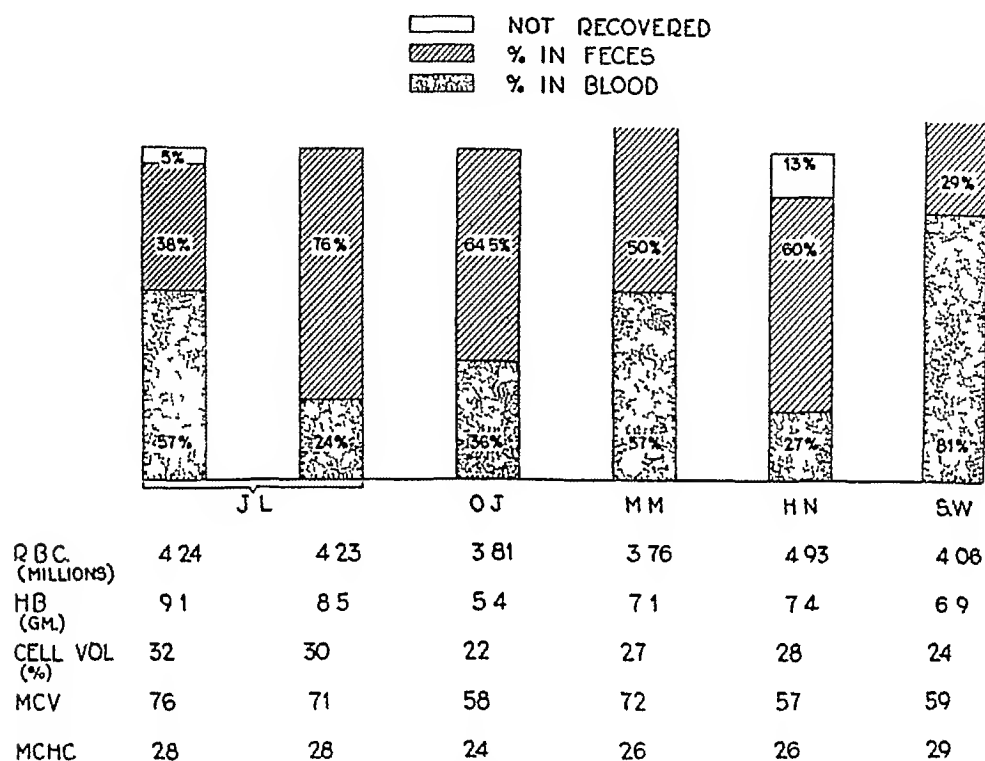


FIG. 1. RECOVERY OF AN ORAL DOSE OF RADIOIRON IN HYPOCHROMIC ANEMIA  
 Dose, 1 mg. Fe per kg. as  $\text{FeCl}_2$

of N NaOH was added. Recovery varied from 91 to 102 per cent, the form in which iron was added did not influence recovery.

Since iron-deficient subjects promptly and quantitatively utilize any iron available to them for hemoglobin synthesis,<sup>1</sup> it was thought that a study of iron absorption in such a group would provide a further means of checking the accuracy of the method. In these patients, if the method is valid, the sum of the radioiron found in the circulating blood and that recovered from the feces should equal the amount given orally in the test dose. Six experiments on patients with hypochromic microcytic anemia were done (table 2, fig. 1). In only one was the recovery less than 95 per cent, this patient vomited four hours after taking the test dose and failed to save the vomitus. It is possible that a small amount of the radioiron was lost in this way. In five experiments, from 95 to 110 per cent of the ingested isotope was recovered in the blood and feces.

These two types of observations define the accuracy of the method. The error



recovering iron from feces may be as great as 10 per cent. The sum of the amount found in circulating hemoglobin plus that recovered from the intestinal tract should not be in error by an amount greater than plus or minus 12 per cent.

## 2. NORMAL SUBJECTS

Absorption of radioiron was measured ten times in eight different normal subjects (table 3, fig. 2). The amount accounted for in blood and feces varied 83-100 per cent of the administered dose, in only two instances was the value less than 90 per cent. Comparison of the amount of radioiron retained by the body (activity in the test dose minus that recovered in the feces) with the amount found in circulating hemoglobin indicates that in 9 of the 10 determinations some iron was

TABLE 3—*The Absorption of Radioactive Iron by Normal Subjects*

Subject	Date	Hematologic Data					Total Test Dose of Radioiron		Radioiron Recovered		
		R B C	Hb	Cell Vol	MCV	MCHC			Feces	Blood	Total
		millions	Gm	%	cu microns	%	mg/kg	counts/min	%	%	%
R D	4-10-46	4.63	14.2	43	93	33	1	600,000	91	7	98
S C	1-6-47	4.10	14.2	42	102	34	1	3,280,000	86	6	92
S C	3-5-47*	4.08	14.4	40	98	36	1	1,630,000	87	10	97
J T	9-16-46	4.82	15.0	44	91	34	1	2,700,000	98	2	100
J T	4-8-47†	4.84	16.3	45	93	36	1	2,540,000	79	4	83
T K.	1-13-47	5.32	17.9	46	87	39	1	3,040,000	83	7	90
C M	1-28-47‡	5.64	16.8	46	82	37	2	3,360,000	88	9	97
G G	2-8-47§	4.80	16.4	47	98	35	1	2,400,000	82	9	91
L F	2-20-47	4.86	16.1	47	97	34	1	2,080,000	81	7	88
J N	8-9-47	4.52	14.2	44	97	32	1	43,520,000	79	11	90

\* Cohn's protein IV-7 (4.5 Gm protein) was given i.v. immediately after the radioiron.

† Cohn's protein IV-7 (14 Gm protein) was given i.v. immediately after the radioiron.

‡ Cohn's protein IV-7 (3.75 Gm protein) had been given i.v. twenty-four hours before the radioiron.

§ G. G. vomited 6 hours after taking the radioiron.

|| The blood volume was determined by the Evans blue dye method.

absorbed but not utilized. Even though this quantity amounted to 17 per cent in the second experiment on J. T. and was 10 per cent or more in three other instances, it was never large. Because of the error of the method, it can only be stated that the results suggest that normal persons absorb more iron than they build into hemoglobin under these conditions.

There are, however, certain conclusions which can be made with assurance. Patients with hypochromic anemia do absorb several times more iron from test doses of this magnitude than do normal persons. On the other hand, normal subjects retain greater amounts than Hahn and his associates originally indicated might be the case.<sup>4</sup> Even though the intestinal mucosa may be one of the principal regulators of iron metabolism as has been suggested, it is not so efficient a regulator that it causes normal subjects to reject iron almost completely.

In three of the experiments listed in table 3, a quantity of the iron-binding globulin of plasma, Cohn's fraction IV-7,\* was given intravenously either before or after the oral test dose of iron. This was done to see whether absorption would be increased if considerable quantities of this globulin were circulating during the period of absorption. The differences were not great enough to justify any conclusion, if there was an increased absorption, the increase was certainly small.

### 3 PATIENTS WITH ANEMIAS OF VARIED ETIOLOGY OR FEVER

Iron absorption has been studied in 12 patients selected because they had diseases in which utilization of absorbed radioiron might well have been incomplete.

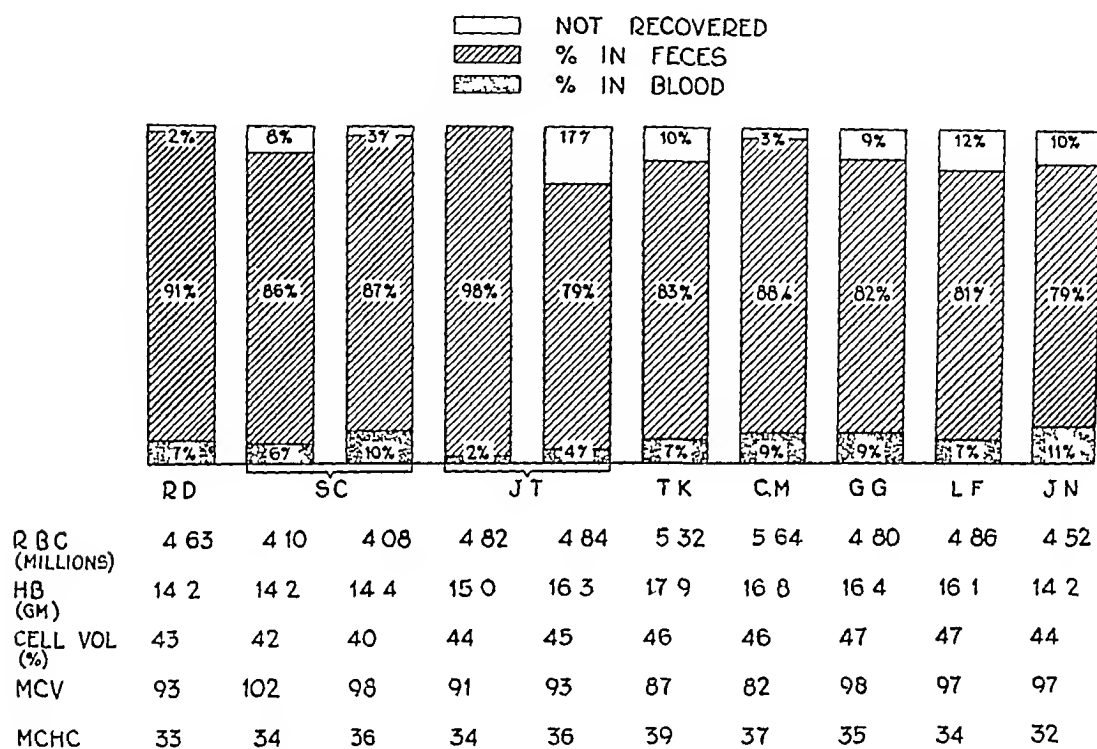


FIG. 2. RECOVERY OF AN ORAL DOSE OF RADIOIRON GIVEN TO NORMAL SUBJECTS  
 Dose, 1 mg Fe per kg as  $\text{FeCl}_2$

(table 4, figs 3, 4) We wished to know (1) how well these persons absorbed iron, and (2) how erroneous the results would have been if the amount of radioiron found in circulating hemoglobin had been taken as the sole measure of absorption. Because of the error in recovering iron from feces, no conclusions can be drawn from the results on patients 5, 10 and 12, even though the amount of radioiron found in their peripheral blood was negligible. For similar reasons, caution is necessary in interpreting the results on patient 6. Patient 12 might have shown greater absorption had she not had unusually rapid intestinal action. Over 60 per cent of the iron given appeared in a stool passed within eighteen hours of the dose. In the remaining observations, however, there is no question about the fact

\* Obtained through the courtesy of Dr. E. J. Cohn.

that more iron was absorbed than could be accounted for in the hemoglobin of circulating blood. Experiments 8 and 9 demonstrate this particularly well. These two patients had Hodgkin's disease associated with fever and hypochromic anemia.

TABLE 4—*The Absorption of Radioactive Iron by Patients with Various Blood Dyscrasias or Infection*

Patient	Diagnosis	Date	Hematologic Data						Total Test Dose of Radioiron		Radioiron Recovered		
			RBC	Hb	Cell Vol	Retics	MCV	MCHC			Feces	Blood	Total
			mil lions	Gm	%	%	cu mi crons	%	mg / kg	counts/ min	%	%	%
1 S McC	Addisonian pernicious anemia	12-6-47	2.04	8.9	26	2.3	127	34	1	3,320,000	77	7	84
2 E B	Addisonian pernicious anemia	1-11-47	2.56	10.8	31.5	2.8	123	34	1	3,232,000	77.5	8	85.5
3 S G	Addisonian pernicious anemia	6-6-47	1.30	5.8	15.5	1.8	119	37	1	4,800,000	84.5	11	95
4 J S	Refractory anemia*	10-1-46	2.86	8.4	25	0	88	34	1	2,220,000	86	0	86
J S	Refractory anemia*	2-18-47	2.35	9.7	28	0	119	35	2	4,050,000	73	0	73
5 M M	Refractory anemia*	4-21-47	2.92	9.2	25	0.7	86	37	1	1,600,000	92	0.2	92
6 W C	Acquired hemolytic anemia	10-3-46	3.25	10.2	33	12.0	102	31	1	2,460,000	84	4	88
7 L B	Sickle cell anemia	8-21-47	3.80	10.7	32		84	34	1	4,800,000	82	2	84
8 D G	Hodgkin's disease	12-13-46	4.34	9.3	32	2.1	74	29	1	3,950,000	48	15	63
9 J W	Hodgkin's disease	4-2-47	3.41	5.4	22	2.8	65	24	1	1,450,000	68	2.5	70.5
10 S D	Diabetic gangrene	4-28-47	5.29	17.1	50		95	34	1	2,000,000	94	0.7	95
11 J C	Hemochromatosis	6-10-47	4.83	16.3	49		101	33	1	5,200,000	78.5	2	80.5
12 C V	Leukemia	8-20-47	1.90	5.8	17		90	34	1	4,496,000	89.5	1	90.5

\* Secondary hemosiderosis from many transfusions

On the basis of fecal recovery, they retained 52 and 32 per cent of the test dose, yet utilized only 15 and 2.5 per cent, respectively, to build hemoglobin.

Particular attention should be directed to the three observations on patients with pernicious anemia. The data for one of these experiments are graphically illustrated in figure 5. Shortly after administration of the test dose of radioiron, specific therapy in the form of liver extract was given. The radioactive isotope

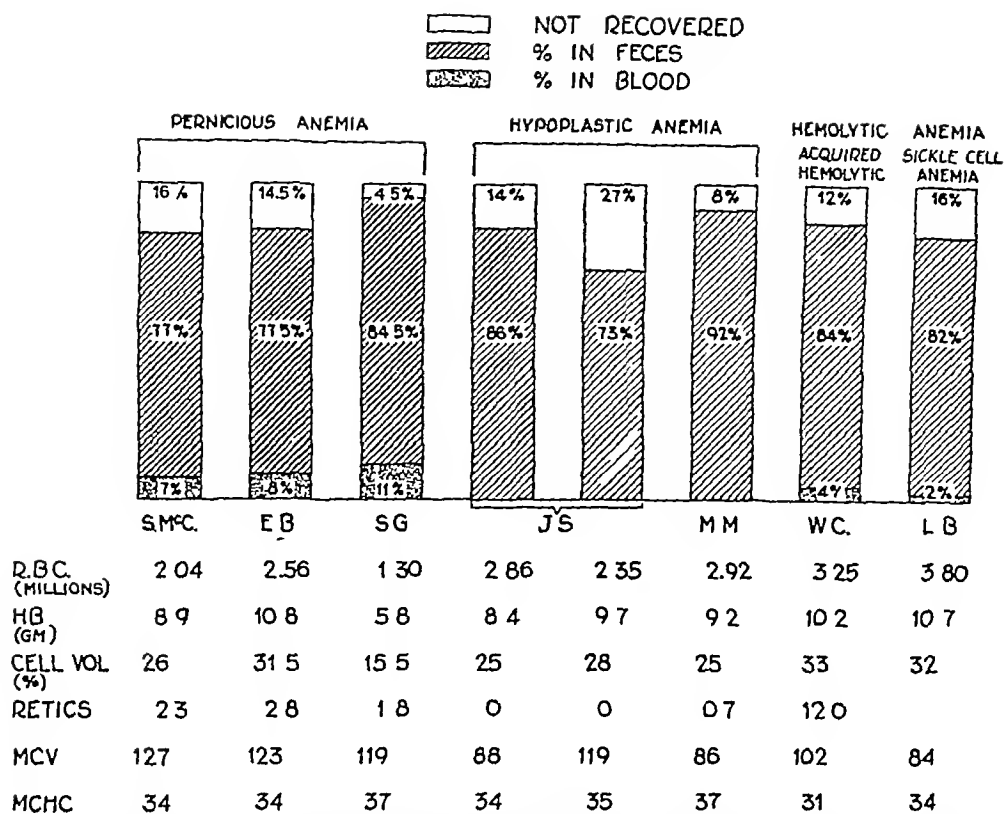


FIG 3 RECOVERY OF AN ORAL DOSE OF RADIOIRON IN ANEMIAS OF VARIED ETIOLOGY  
Dose, 1 mg Fe per kg as  $\text{FeCl}_2$

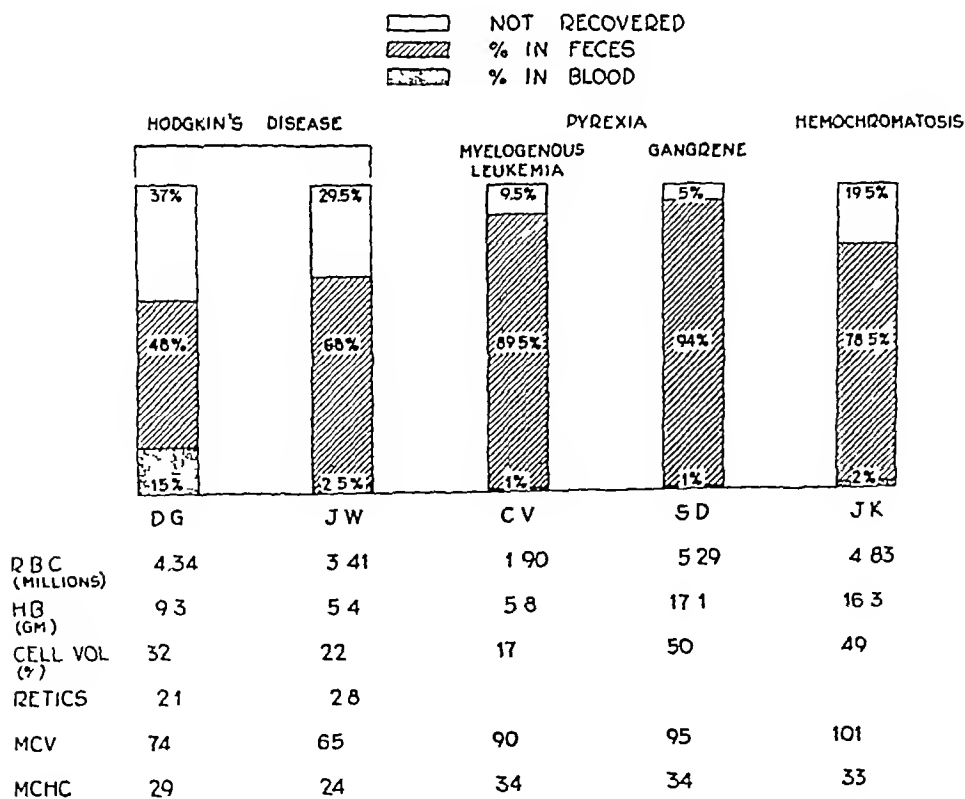


FIG 4 RECOVERY OF AN ORAL DOSE OF RADIOIRON IN PYREXIA AND IN HEMOCHROMATOSIS  
Dose, 1 mg Fe per kg as  $\text{FeCl}_2$

SG ♂ 58 yrs of age Addisonian Pernicious Anemia  
Histamine Refractory Achlorhydria

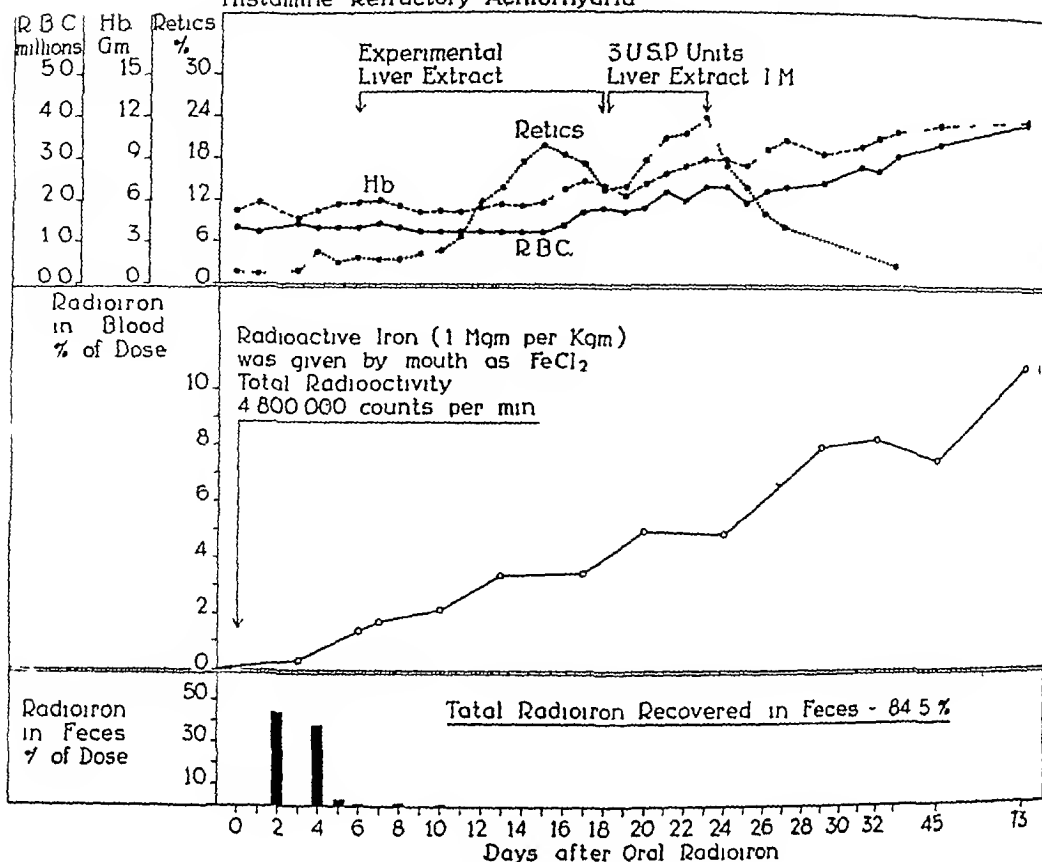


FIG 5 Absorption of Radioactive Iron by a Patient with Pernicious Anemia

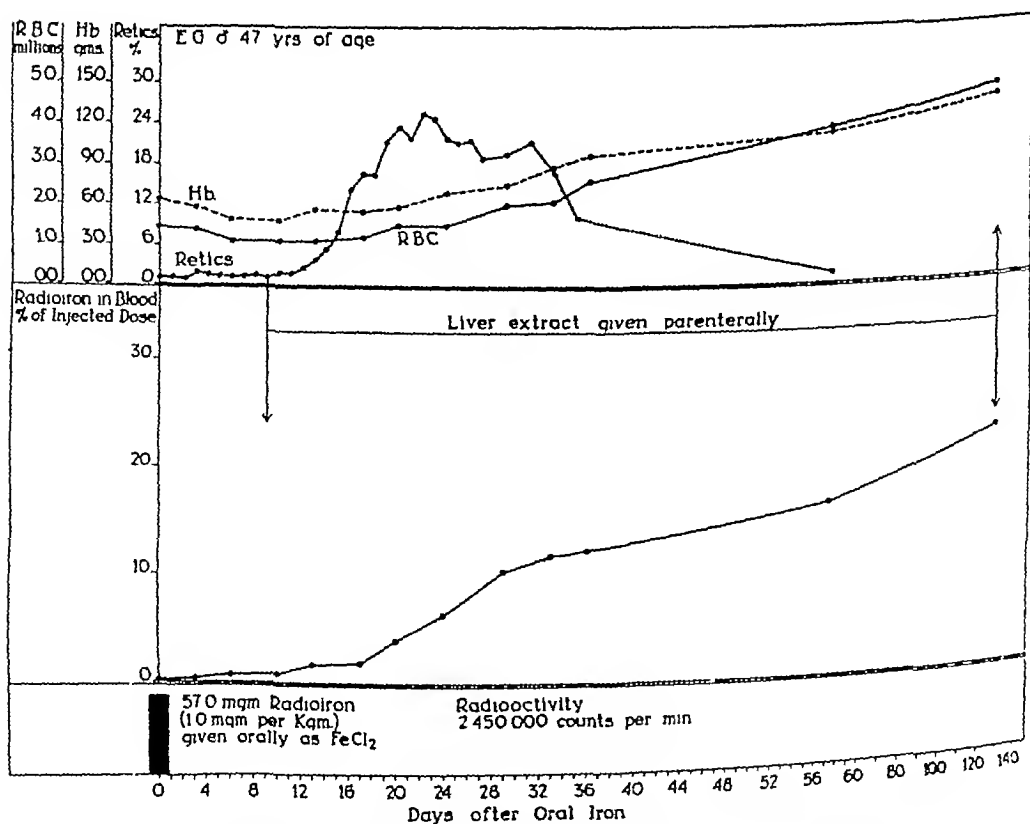


FIG 6 Absorption of Radioactive Iron by a Patient with Pernicious Anemia

appeared slowly in this man's blood during a period of several months as he recovered from his anemia. If the observations had been stopped at ten days, it would have been concluded from measurement of isotopic hemoglobin that only 2 per cent of the test dose had been absorbed, yet 11 per cent eventually appeared in the blood. An even more dramatic result is illustrated in figure 6, data for this experiment are not included in table 4 because fecal recovery was not obtained. This patient had enough iron stored in his tissues to raise his hemoglobin from 7 to more than 12 grams per 100 cc. after specific therapy was begun, yet he absorbed more than 20 per cent of a test dose of radioiron. This fact was completely masked

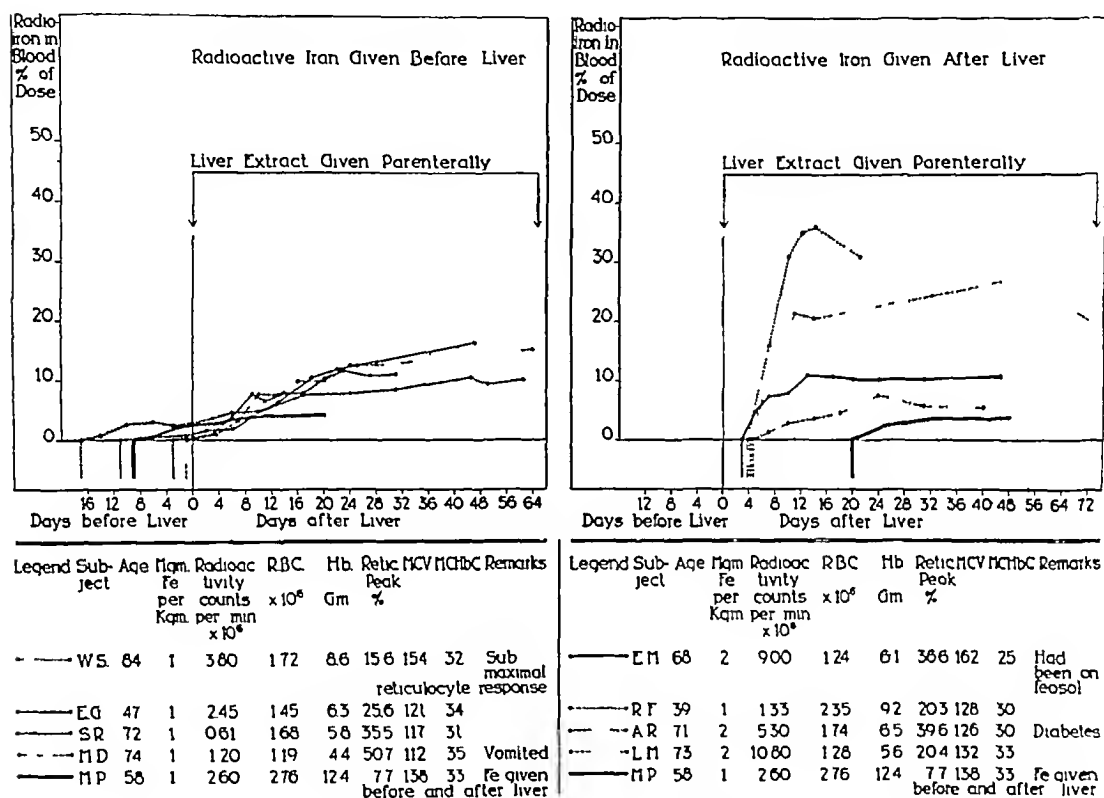


FIG. 7. Absorption of Radioactive Iron by Patients with Pernicious Anemia

during the period of relapse and became evident only as he recovered from his anemia. Additional observations of a similar nature are recorded in figure 7. When the radioiron was given after therapy with liver extract, absorption was occasionally greater than during the pretherapy period.

Of particular interest also is the retention of over 20 per cent of the test dose by a patient with hemochromatosis while only 2 per cent appeared in his blood. Even if the fecal recovery was low by 10 per cent, he still absorbed five times as much as he built into hemoglobin. This man had diabetes, hepatomegaly, and bronzing of his skin. He had never received any transfusions to account for secondary hemosiderosis. The diagnosis was confirmed by biopsy of both liver and skin.

#### 4. EFFECT OF LARGE DOSE OF INERT IRON GIVEN INTRAVENOUSLY ON ABSORPTION

To one of the patients with hypochromic anemia (J. L., table 2), 1345 mg. of inert iron as colloidal ferric hydroxide were given intravenously. This amount was

sufficient to increase his hemoglobin level from about 9 to over 12 grams per 100 cc. Twenty-one days prior to this injection, the patient absorbed 57 per cent of a standard test dose of radioiron (figure 8). Two days after the intravenous therapy, before he had converted much of the ferric hydroxide to hemoglobin and at a time when his tissues contained more than a gram of iron, he absorbed 24 per cent of a second test dose. Retention was less, therefore, after his tissues were well supplied with iron, but was still relatively large.

A similar result was obtained in another patient with hypochromic anemia; these data are not listed in table 2 because fecal recoveries were not done. This woman

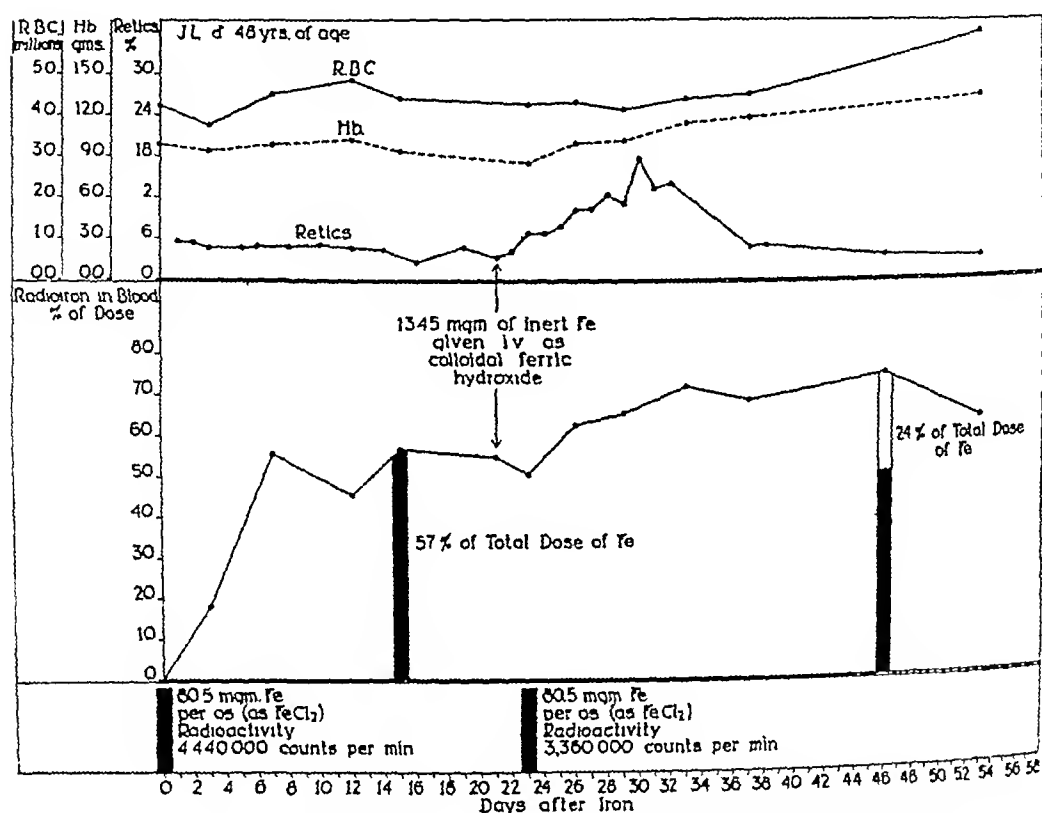
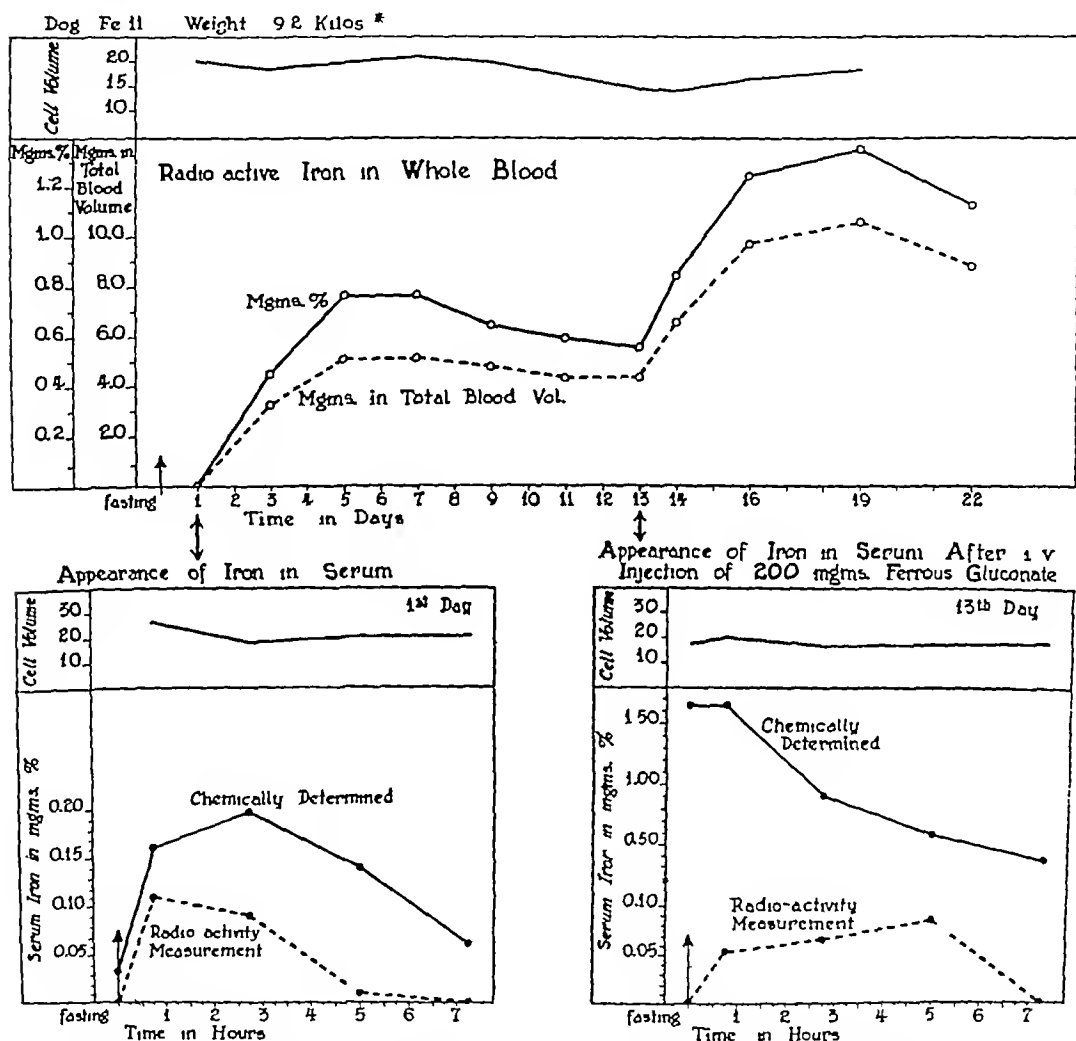


FIG. 8 The Effect of a Large Dose of Parenterally Administered Iron on Iron Absorption

(A. D.), with a hemoglobin value of 9.4 Gm and a mean corpuscular hemoglobin concentration of 29 per cent, absorbed 60 per cent of a test dose of radioiron in a control experiment. Six weeks later she was given intravenously 755 mg of iron as colloidal ferric hydroxide. On the eleventh day after this procedure, when her hemoglobin had risen to 11.3 Gm and the mean corpuscular hemoglobin concentration was 30 per cent, she was given a second dose of radioiron. Of this, 27 per cent appeared in her blood as hemoglobin. Likewise, when 480 mg of iron as colloidal ferric hydroxide were injected into a dog with depleted iron stores, absorption was decreased from 14.9 per cent (control period) to 6.9 per cent. These results are at variance with those of Hahn and his associates<sup>8</sup> who reported in one experiment that colloidal iron (304 mg) given by vein to an anemic dog did not significantly modify iron absorption.

In other experiments done on dogs made chronically anemic by regular phlebotomy, the authors have demonstrated that the level of iron in the serum has no apparent effect on iron absorption. If the serum iron level was raised above 500 mg per cent by intravenous administration of a soluble iron salt immediately before radioiron was given orally, absorption was not decreased (figure 9)



\* Given 4 mgms. radio active iron per kilogram body weight (as ferrous chloride). (1,459,000 counts per hour)

FIG. 9 Effect of Iron in Blood Serum on Iron Absorption Radioactive Isotope Used

### DISCUSSION

The results of these experiments indicate clearly that patients with impaired hemoglobin formation are capable of absorbing more iron than is used for hemoglobin synthesis. The data also suggest, but do not prove, that normal subjects may occasionally absorb more iron than is built immediately into hemoglobin. Caution must be exercised, therefore, in interpreting estimates of iron absorption obtained solely by measuring the per cent of a given test dose of radioiron which appears in the circulating blood as hemoglobin. Even when these limitations are



recognized, the isotope method is still the best one available for studies of iron absorption. Recovery of unabsorbed iron from feces, as was done in these studies, is too difficult and too time consuming to be practical. An error of approximately 10 per cent, furthermore, is involved in the recovery. If a study is to be made of the relative absorption from different iron salts, or of the effect of various influences like achlorhydria, food, the calcium-phosphorus ratio, etc., on iron absorption, the ideal procedure would be to use the isotope technic but to select only afebrile, iron-deficient patients as test subjects.

Objection might possibly be made to the experiments reported in this paper on the ground that one cannot be sure that a portion of the iron recovered in feces had not been absorbed and promptly excreted into the colon. There is abundant evidence, however, that the amounts of iron excreted into the gastrointestinal tract are minute.<sup>14 15</sup> Any error introduced in this manner would be much less than the error of recovery per se and would be insignificant.

These results are of theoretic interest chiefly as they relate to the theory that the intestinal mucosa serves as a major regulator of iron metabolism.<sup>8 16 17</sup> Granick has postulated the following explanation of absorption: the intestinal mucosal cells contain a protein, apoferritin, which combines with iron to form ferritin. The ferritin iron is thought to be in equilibrium with small amounts of ferrous ions in the cells, and the ferrous ions in turn are postulated as being in equilibrium with the iron in plasma. According to this concept, iron is taken up by mucosal cells until all the apoferritin is converted into ferritin. No more is absorbed until some of the ferritin has given up its iron to plasma. This theory explains beautifully the fact that iron-deficient subjects absorb more iron than do normal persons. It does not account adequately, however, for the equally clear demonstration that patients with pernicious anemia in relapse, with refractory anemia, or with hemolytic anemias may also absorb fairly large amounts of the metal even though their tissues are replete with iron. If the intestinal mucosa is a major regulator of iron metabolism, protecting the body from an uptake sufficiently great to cause toxic concentrations in the tissues, it at least is not as complete a regulator as it was first thought to be.

Many of the factors which control iron absorption are unquestionably still unknown. In unpublished experiments, the authors have confirmed Hahn's observations that anemia by itself does not influence iron absorption. Uptake from the intestinal tract has been shown to be independent of the plasma iron concentration. On the other hand, if tissue iron reserves of subjects with hypochromic anemia are partially restored by the parenteral administration of large amounts of iron, uptake of the metal from the alimentary tract becomes less complete. The possibility has been explored that a factor may be present in the blood of iron-deficient subjects which stimulates absorption, but in two unpublished experiments the infusion of large amounts of plasma from iron-deficient into normal dogs has failed to affect the quantity absorbed. The mucosal block theory of Hahn and Granick remains the best explanation for all the known facts about iron absorption, but the 'block' should be thought of in relative terms.

## SUMMARY AND CONCLUSIONS

- 1 The isotope technic for studying iron absorption has been extended to measure the unabsorbed isotope in feces as well as the amount synthesized into hemoglobin. The recovery of radioiron from feces was shown to be accurate within 10 per cent.
- 2 There was suggestive evidence to indicate that, with the 1 mg per kilogram dose employed, normal subjects may sometimes absorb more iron than is converted within a two week period into hemoglobin.
- 3 Patients with fever, untreated pernicious anemia, and refractory anemia were shown to absorb more iron than they use for hemoglobin.
- 4 Patients with hemolytic anemia may absorb more iron than can be recovered in the peripheral blood at any one time because isotopic hemoglobin is removed from the circulation at a rapid rate.
- 5 Except in afebrile patients with hypochromic anemia, acceptance of the per cent of a given dose of radioiron which appears in circulating hemoglobin as a measure of iron absorption must be made with caution.
- 6 The theory that mucosal cells accept iron for absorption or block its assimilation provides the best known explanation for iron absorption, but patients with adequate iron stores may assimilate considerable quantities of the metal and the block must be regarded as relative.

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# OSMOMETRIC BEHAVIOR OF NORMAL AND ABNORMAL HUMAN ERYTHROCYTES

By GEORGE M. GUEST, M.D.

VARIOUS investigators have postulated two main principles which seem to govern the swelling and hemolysis of red blood cells suspended in hypotonic salt solutions—namely, the cells imbibe water according to the laws of osmosis, and their maximum swelling is limited by an inelastic surface membrane. According to this concept, hemolysis occurs when the erythrocytes in hypotonic solutions attain a critical volume at which the cells rupture and allow hemoglobin to escape. Studies of the physico-chemical mechanisms involved in the processes of osmotic swelling and hemolysis of erythrocytes have involved a great deal of controversy, especially over the question of whether the mammalian red cell behaves as a 'perfect osmometer,' adapting itself to changes in osmotic pressure of surrounding fluids by the transfer of water alone (Ponder<sup>18</sup>). Evidence assembled by Ponder<sup>19</sup> from many sources indicates that the erythrocytes of various species sometimes do behave as perfect osmometers, but at other times and under varying conditions they behave as decidedly imperfect osmometers. The reasons for this variable behavior are still obscure.

Methods devised for the measurement of the osmotic fragility of red cells have had clinical applications especially valuable in the diagnosis of congenital hemolytic jaundice and in the differential diagnosis of other types of hemolytic disease. The first development of such a method is generally ascribed to Hamburger<sup>10</sup> who in 1883 devised a test that involved the notation of 'beginning' hemolysis and 'complete' or total hemolysis, or the points of minimal and maximal resistance of red cells suspended in a series of solutions of diminishing concentrations of sodium chloride. Jolly<sup>16</sup> states that even earlier, in 1880, Chanel<sup>2</sup> described a method for measuring red cell fragility in hypotonic salt solutions and applied it in clinical studies. It is of interest to note that Chanel's was a quantitative test, in contrast to the essentially qualitative test of Hamburger. According to Jolly, Chanel made suspensions of blood cells in a series of salt solutions of diminishing concentrations and after a given time determined the number of cells destroyed by counting the intact cells. By this method he demonstrated diminished osmotic resistance of erythrocytes in certain anemias and increased resistance in certain types of jaundice. Whitby and Hynes<sup>34</sup> independently developed approximately the same procedure in 1935. Several refinements of Hamburger's procedure have been suggested to disclose minor differences in cell fragility that characterize certain pathologic types of red cells (Wintrobe<sup>25a</sup>), but the noteworthy advances in methodology are mainly confined to quantitative measurements of hemolysis. Estimations of the degree of hemolysis at each point in the hemolytic series have

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been made by determinations of the hemoglobin liberated from the erythrocyte into the suspension fluids by visual colorimetry<sup>1 11</sup> and by more accurate photoelectric colorimetry<sup>12 23</sup>

Various technics have been employed for demonstrating the swelling, or osmometric behavior, of various species of red cells in hypotonic fluids: determinations of cell volume have been made by centrifugation, employing several types of hematocrit apparatus, and by electrical conductivity measurements; estimations of mean cell diameters have been made by diffraction methods and by measurements of projected images of the cells, sometimes with photography (Ponder<sup>17-21</sup>). Another approach to this study is by kinetic methods (Jacobs, et al.<sup>13-15</sup>) whereby the rate of hemolysis of red cells is determined, within periods of a few seconds, in hypotonic solutions at the critical tonicities, or less, that produce complete hemolysis. Such technics have been used rarely if at all in clinical studies of abnormal types of erythrocytes.

Seeking a practical method for studying the osmometric behavior of red cells in clinical investigations, Guest and Wing<sup>5</sup> at first employed standard Van Allen hematocrit tubes<sup>22</sup> in which blood was diluted in the usual series of salt solutions. After centrifugation the volume of packed cells, measured in the graduated stems of the pipets, afforded an estimate of the swelling of the cells that occurred before hemolysis, and the progress of hemolysis at lower tonicities was judged by the diminishing mass of cells as well as by the color of the fluid in the bulbs of successive pipets. A later elaboration of that procedure<sup>6</sup> combined the quantitative determination of hemolysis with measurements of the volume of cells at each stage of the test. Observations made by this method on the erythrocytes of normal adults, children and newborn infants indicated that the swelling of these normal human red cells in every instance followed closely that expected in perfect osmometers. Moreover, the maximum volumes attained by the normal cells before hemolysis agreed closely with predictions based on estimations of their mean surface area, thus supporting the view that the red cell cannot distend beyond the limit set by its surface area. The present paper reports further data, gathered by that method, on the erythrocytes of normal subjects and on the erythrocytes of patients suffering several diseases in which abnormal fragility of red cells is commonly found.

## METHOD

Only the main steps of the procedure and the principal calculations employed need be given here; further details may be found in the paper by Guest and Wing.<sup>6</sup> The special Van Allen pipet with elongated bulb devised for this method is calibrated to contain 8.0 cc. when filled to the upper mark, and to contain 0.02 cc. in the graduated portion of the stem marked with 100 divisions. Twelve or 25 pipets are usually used in each test. Heparinized blood is drawn into the stem to the 100 mark, and after it the appropriate solution of NaCl is drawn into the pipet almost to fill the bulb, leaving a small air bubble to facilitate mixing the fluid and blood. The usual series of salt solutions is employed, beginning with 0.9 per cent NaCl, with decrements in concentrations of 0.1 or 0.05 per cent before the beginning of hemolysis, and 0.025 per cent through the expected critical range of hemolysis. The tip of each pipet is then sealed with a rubber cushioned spring clip and the pipets are left resting in a horizontal position at room temperature an hour, after which they are centrifuged 20 minutes at 2500 to 3000 r.p.m., and the volume of packed cells is read in per cent on the graduated stem. The bulb is then filled to the 8.0 cc. mark with distilled water (making the dilution of blood 1:400), the fluid is transferred by means of a long stemmed

pipet to colorimeter tubes, and the hemoglobin content of each fluid is read in a photoelectric colorimeter.<sup>3</sup> The total hemoglobin content of the blood is determined on a separate sample measured into distilled water, with a drop of ammonium hydroxide added to insure complete hemolysis and absence of turbidity. The hemoglobin content of each supernatant fluid in the series is then expressed in per cent of the total hemoglobin, indicating the per cent hemolysis found at each tonicity. Further calculations are made to compare the observed cell volumes with the expected osmometric swelling at each tonicity and to predict the maximum swelling expected if the cells attain the volume of spheres within the limits of their estimated mean surface area.

TABLE 1—*Hemolysis and changes in volume of normal erythrocytes of blood diluted 1:400 in solutions of sodium chloride. Normal child, S. S.*

These data are presented graphically in figure 1.

NaCl	Hemolysis	Volume of cells				R value†
		Readings	Uncorrected for hemolysis	Corrected for hemolysis	Expected osmometric volume*	
%	%	%	%‡	%‡	%‡	
0.900	0	38.2	100		100	
0.800	0	41.2	108		109	0.99
0.700	0	44.5	117		120	0.97
0.600	0	49.6	130		135	0.96
0.550	0	52.2	137		145	0.95
0.520	0.6	55.2	145	146	150	0.97
0.500	1.1	58.0	152	154	156	0.99
0.475	2.4	62.0	162	166	163	1.02
0.450	4.3	61.5	161	168	170	0.99
0.425	19.8	53.0	139	173	178	0.97
0.400	50.4	31.0	81	164	188	
0.375	79.0	9.2	24	115	198	
0.350	88.9	2.0	5		210	
Average						0.98

\* Volume of a perfect osmometer, in per cent of its initial volume, assuming the initial water content to be 70 per cent by volume.

† R value is the ratio, observed cell volume (corrected for hemolysis) to the expected osmometric volume.

‡ Values in per cent of the initial volume of the cells in 0.9 per cent NaCl solution.

Data thus determined on a sample of normal blood are presented in table 1. Column 1 indicates the tonicities at which the readings of hemolysis, listed in column 2, and of cell volume, listed in column 3, were made. In column 4 the values for cell volume at the respective tonicities are converted to percentages of the initial volume as read in the 0.9 per cent NaCl solution, and in column 5 these values in turn are corrected for hemolysis (employing the values in column 2) to indicate the true volume, or swelling, of the cells remaining intact in the tubes where partial hemolysis occurred. The values for expected osmometric volume, given in column 6, are based on the formula of Ponder<sup>10</sup>  $V = W(1 - T) + 100$ , where  $V$  = the new volume of the cells in per cent of their initial volume,  $W$  = the percentage by volume of water in the cells, taken as 70 per cent,  $T$  = the ratio of the tonicity of

the medium to 0.9 per cent NaCl solution (i.e., with 0.45 per cent NaCl,  $T = 0.5$ ), 100 = the initial volume of the cells. In the last column the "R values" represent

TABLE 2—*Osmotic swelling and hemolysis of normal human erythrocytes in hypotonic saline solutions*

Normal Subjects	Initial Mean Dimensions				Volume of a sphere with same surface area	Tonicity at which maximal swelling occurred	Hemolysis at this tonicity	Expected osmometric volume at this tonicity*	Maximum Cell volume		R value
	Volume	Diameter	Thickness	Surface area					Expected, calculated from surface area	Found, observed volume corrected for hemolysis	
	$\mu^3$	$\mu$	$\mu$	$\mu^2$	$\mu^2$	% NaCl	%	%	%†	%†	
Adults											
1	92	7.7	1.98	141	157	0.450	2.9	170	171	172	1.00
2	90	7.7	1.93	140	156	0.425	24.4	178	173	179	1.00
3	98	7.8	2.05	146	166	0.450	32.0	170	169	171	0.96
4	98	7.8	2.05	146	166	0.475	37.9	163	169	166	0.99
5	91	7.7	1.95	140	156	0.425	47.6	178	172	180	1.01
6	87	7.6	1.92	137	151	0.450	11.6	170	172	172	1.03
7	86	7.6	1.94	137	151	0.450	16.7	170	176	178	1.03
8	80	7.4	1.86	129	138	0.450	19.3	170	172	172	1.01
9	94	7.8	1.97	144	162	0.450	26.0	170	172	174	1.02
10	88	7.7	1.89	139	154	0.425	22.1	178	175	175	0.97
11	89	7.4	2.07	134	146	0.450	35.0	170	164	165	1.01
12	89	7.3	2.13	133	144	0.475	26.3	163	162	166	1.01
13	98	7.8	2.05	146	166	0.475	18.0	163	166	165	1.00
Children											
14	82	7.6	1.81	134	146	0.425	14.6	178	178	174	0.99
15	77	7.4	1.79	128	136	0.425	14.4	178	177	172	0.97
16	84	7.5	1.90	133	144	0.425	14.8	178	174	173	0.99
17	82	7.5	1.86	132	143	0.425	19.8	178	174	173	0.97
18	77	7.4	1.79	128	136	0.425	18.3	178	177	174	0.99
Newborn infants											
19	109	7.7	2.34	150	173	0.475	10.9	163	159	153	0.95
20	110	8.1	2.14	157	185	0.450	21.0	170	168	165	0.95
21	113	8.0	2.25	157	185	0.450	22.7	170	164	163	0.96
22	110	7.9	2.24	154	179	0.450	10.8	170	163	162	0.93
23	106	7.9	2.16	152	176	0.425	42.8	178	166	168	0.96
24	104	7.9	2.12	151	174	0.425	22.3	178	167	168	0.95

\* Volume of a perfect osmometer, in per cent of its initial volume, assuming the initial water content to be 70 per cent by volume.

† Values in per cent of the initial volume of cells in 0.9 per cent NaCl solution.

R value in each case is the average for all points determined in the series through the point of maximum swelling of unhemolyzed cells.

the ratio of the observed/expected swelling. Ponder introduced the factor R in the formula,  $V = RW(1/T - 1) + 100$ , "to reconcile observation with theory," after

Initial Mean Dimensions							

Subjects	Initial Mean Dimensions				Volume of a sphere with same surface area	Tonicity	Hemolysis at this tonicity	Expected osmotic volume at this tonicity*	Maximum Cell Volume		R value
	Volume	Diameter	Thickness	Surface area					Expected, calculated from surface area	Found, observed volume corrected for hemolysis	
Congenital spherocytosis											
P H	85	7.0	2.21	126	133	% NaCl	%	%	%†	%†	1.00—Av
H A	77	6.8	2.12	118	120	0.500	9.0	156	156	156	1.00—Av
P A	85	6.4	2.64	117	119	0.525	1.0	150	156	154	0.99—Av
J J	75	6.6	2.22	114	114	0.575	23.0	140	156	152	1.01—Av
J J	77	6.4	2.39	113	112	0.550	16.5	145	140	138	0.98—Av
P J	74	6.3	2.33	108	105	0.600	16.6	135	145	152	0.97—Av
M J	79	6.2	2.62	111	110	0.550	44.9	145	145	128	1.00—Av
D J	72	6.3	2.31	108	105	0.600	46.7	135	142	138	0.98—Av
W M	82	7.0	2.13	124	130	0.525	13.9	150	139	135	0.97—Av
W M	78	7.3	1.81	125	132	0.525	14.3	150	146	148	1.00—Av
						0.450	43.7	170	159	153	0.98—Av
								174	168	168	1.02—Av
Hypochromic anemia											
F D	75	7.8	1.57	134	146	0.375	24.3	198	195	199	1.00—Av
F H	71	7.2	1.74	121	125	0.450	29.2	170	176	171	1.04—Av
B M	63	6.9	1.69	111	110	0.450	31.7	170	175	169	1.00—Av
R G	57	6.5	1.72	102	97	0.475	9.3	163	175	161	1.00—Av
D P	55	6.8	1.51	105	101	0.350	69.7	210	170	167	0.80
L W	50	6.6	1.46	99	93	0.450	34.0	163	165	166	1.02—Av
						0.400	50.0	188	166	159	0.87
						0.500	28.6	156	155	155	1.04—Av
						0.450	48.9	170	186	155	0.91
Sickleemia											
M J W	65	7.9	1.33	131	141	0.400	1.0	188	165	165	0.88—Av
M W	79	8.0	1.57	140	156	0.275	15.7	260	219	219	0.85
E J	89	7.8	1.87	141	157	0.600	1.5	135	124	124	0.92—Av
						0.350	42.0	210	156	137	0.74
						0.525	1.3	150	137	137	0.91—Av
						0.375	30.8	198	178	178	0.90
Thalassemia											
G B	94	9.1	1.45	171	210	0.500	1.0	156	138	138	0.88
J B	62	7.4	1.45	120	124	0.275	18.0	260	213	213	0.81
						0.375	21.0	198	199	199	0.98—Av
Pernicious anemia											
J W	153	9.7	2.07	211	288	0.500	1.0	156	138	138	0.88
						0.275	18.0	260	213	213	0.81
						0.375	21.0	198	199	199	0.98—Av

\* Volume of a perfect osmometer, in per cent of its initial volume, assuming the initial water content to be 70 per cent by volume

† Values in per cent of the initial volume of cells in 0.9 per cent NaCl solution

R values.  $\bar{A}_v$  represent the average for all points in the series up to the top city indicated, other values are for the one point indicated e.g., for the patient R. G., with hypochromic anemia the average of R values for tonicities 0.8 to 0.775 per cent NaCl was 1.00 but at 0.350 per cent maximal swelling of the cells occurred, the R value was 0.50



finding discrepancies between observed volumes and expected volumes of cells at different tonicities, when some erythrocytes did not behave as perfect osmometers. Where the observed swelling agrees with that calculated from the formula, with the water content of the cells assumed to be 70 per cent, the  $R$  value is 1.00. The  $R$  values at different points in the series range between 0.95 and 1.02, a degree of variability that may be regarded as approximately the experimental error of the method.

Values for the mean volume of the cells in the undiluted heparinized blood were calculated as usual from the hematocrit measurement<sup>6</sup> and the cell count, and the

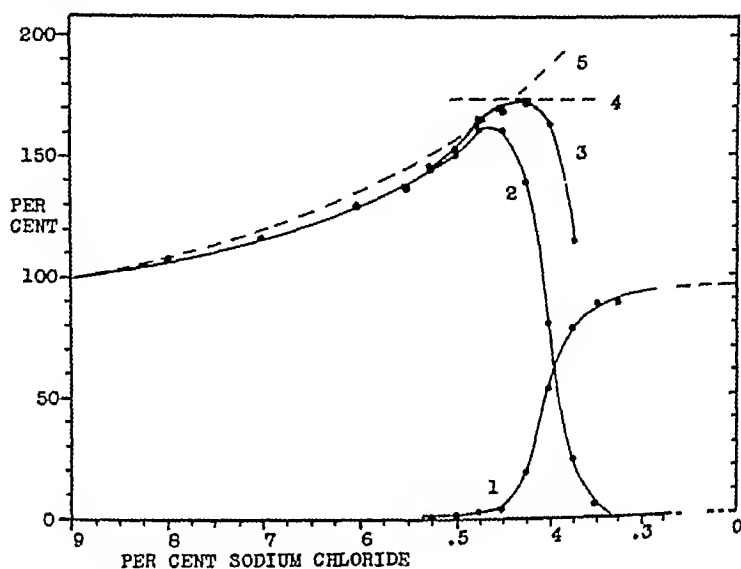


FIG. 1. OSMOTIC BEHAVIOR OF NORMAL ERYTHROCYTES IN THE BLOOD OF A HEALTHY CHILD, S. S., 3 YEARS OF AGE

Hemolysis and changes in volume of erythrocytes in hypotonic salt solutions (1) Hemolysis, expressed in per cent of the total hemoglobin in the sample, (2) Observed cell volume, per cent of the initial volume of the cells in 0.9 per cent NaCl solution, (3) Cell volumes corrected for hemolysis, i.e., the volume of the unhemolyzed cells, (4) Expected maximal cell volume calculated from the mean surface area, (5) Expected osmometric volume of the cells at different tonicities, assuming the initial water content of the cells to be 70 per cent. Data on this blood are listed in table 1.

mean diameter of the cells was read from stained films by means of the Haden Hauser erythrocytometer.<sup>11</sup> The mean thickness ( $T$ ) was calculated from the mean volume and diameter ( $T = V/\pi r^2$ ). The mean surface area was calculated as that of a flat disc of the same thickness and diameter. The expected maximal swelling was calculated as the percentile relationship of the volume of a sphere with the same surface area to the initial mean volume of the cells, i.e., the percentile swelling was expected if the cells attain their maximal volume as spheres within the limits of their mean surface area, following essentially the steps suggested by Haden.<sup>7, 8</sup>

#### OBSERVATIONS

Several different patterns of osmotic behavior of human erythrocytes are illustrated by examples cited in the accompanying tables and figures, which are set

lected from a large series of studies that were made during the years 1939 to 1943 with the assistance of Miss Mary Wing Presumably normal red cells from healthy

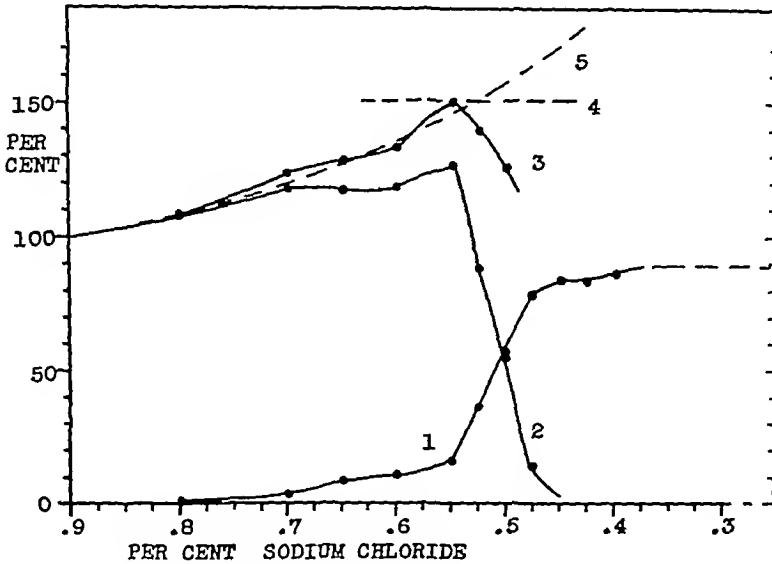


FIG 2 CONGENITAL SPHEROCYTOSIS, OR HEMOLYTIC ICTERUS, IN A BOY, J J , 4 YEARS OF AGE  
For explanation of symbols see figure 1

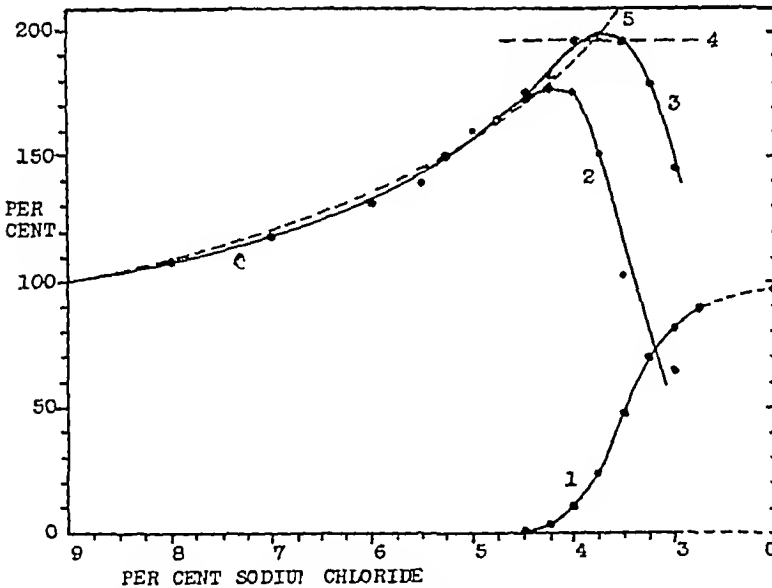


FIG 3 MILD ANEMIA, WITH HYPOCHROMIA AND MICROCYTOSIS, PRESUMABLY DUE TO SIMPLE IRON DEFICIENCY, IN A WOMAN, F D  
For explanation of symbols see figure 1

subjects are represented in tables 1 and 2 and figure 1, abnormal types of cells are represented in table 3 and in figures 2 to 7

*Normal subjects* Observations on the blood of a normal child, S S are illustrated by figure 1, and by data listed in table 1 and in table 2, example 17 In the figure, curves 2 and 3 show how the swelling of the cells before and after the point

of beginning hemolysis (curve 1) followed closely the expected osmometric volume (curve 5) at each tonicity, to reach exactly the expected maximal cell volume (horizontal line 4) predicted from the mean surface area calculated from the measurements of initial dimensions

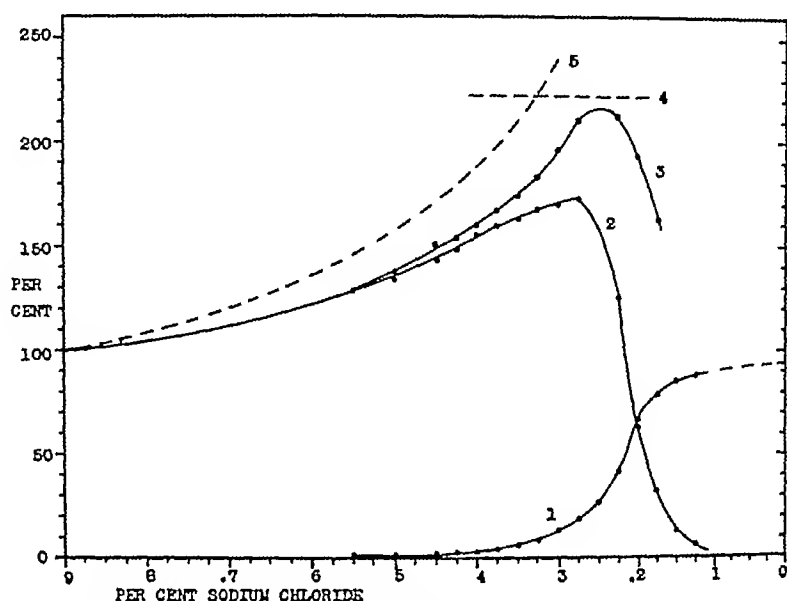


FIG 4 THALASSEMIA (COOLEY'S DISEASE, OR MEDITERRANEAN ANEMIA) IN A BOY, G. B., 15 YEARS OF AGE

For explanation of symbols see figure 1

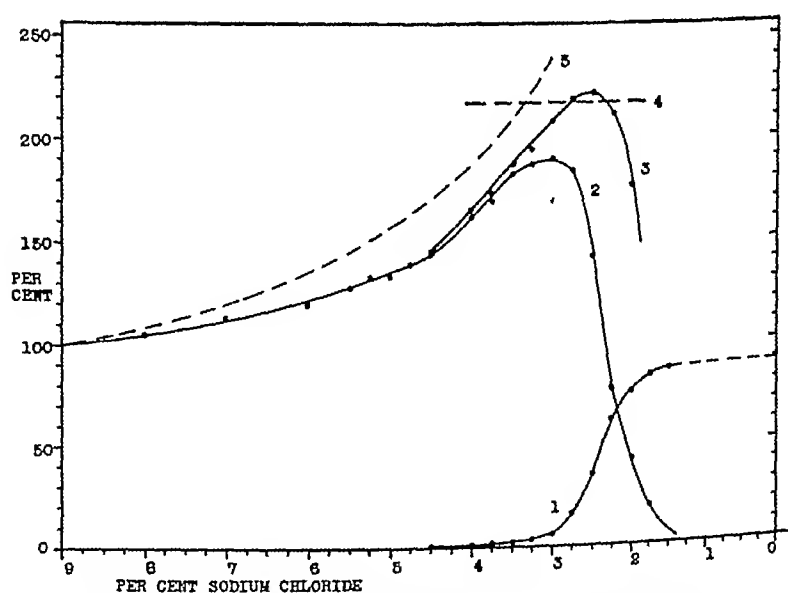


FIG 5 SICKLELEMA IN A NEGRO BOY, M. J. W., 10 YEARS OF AGE

For explanation of symbols see figure 1

In table 2 data on the erythrocytes of normal subjects, adults, children and newborn infants, are arranged to show how normal cells, though varying considerably in their dimensions of mean volume, diameter and thickness, conform closely to the pattern of behavior illustrated in figure 1. Normal erythrocytes usually attain a maximal volume of from 170 to 175 per cent of their initial volume, and this degree

of swelling is found most often in about 0.425 per cent NaCl solution. The  $R$  values in table 2 represent the average of all values determined in the series for each blood up to the point of maximal swelling. Among the bloods from adults and

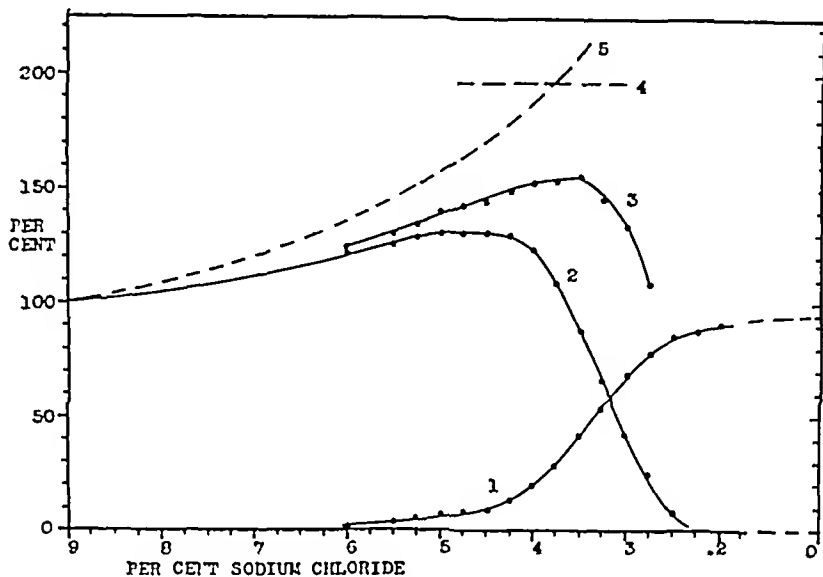


FIG 6 SICKLEMIA WITH ANEMIA IN A NEGRO BOY, M W, 4 YEARS OF AGE

For explanation of symbols see figure 1

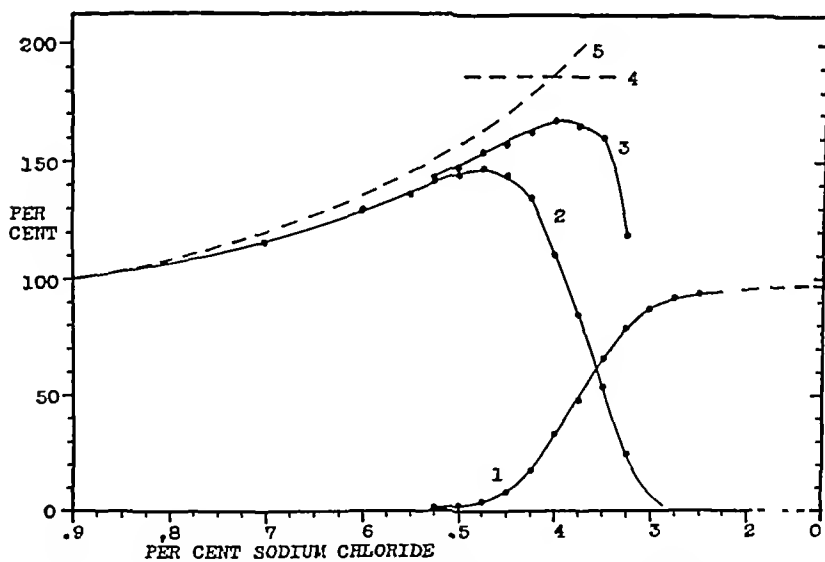


FIG 7 PERNICIOUS ANEMIA IN A MAN 70 YEARS OF AGE, WITH RETICULOCYTOSIS SHORTLY AFTER TREATMENT WITH LIVER EXTRACT

For explanation of symbols see figure 1

children listed, the deviations of the  $R$  values from the theoretically expected value of 1.00 are scarcely more than can be ascribed to experimental error. It is to be noted that the  $R$  values for the macrocytic cells of newborn infants were consistently slightly low, around 0.95, but the observed maximal swelling agreed closely with the values, around 165 per cent, predicted from the mean surface area

*Congenital spherocytosis* Figure 2 illustrates diagrammatically the osmometric behavior of the erythrocytes of a 4 year old boy (sample J J , in table 3) suffering typical manifestations of congenital hemolytic jaundice. Hemolysis of these cells began in 0.8 per cent NaCl solution and appeared to be complete, with cellular residue too low to be read, in 0.45 per cent solution (although the hemoglobin liberated in this solution represented only 85 per cent hemolysis). The swelling of the unhemolyzed cells followed fairly closely the expected osmometric volume, and reached exactly the calculated maximum volume, 152 per cent of the initial cell volume.

In table 3 are listed data on ten samples of bloods from patients suffering congenital hemolytic jaundice. Hemolysis of the cells in these bloods began in solution of NaCl varying from 0.85 to 0.65 per cent, and in all instances appeared to be complete (by visual inspection) in solutions around 0.40 per cent. Except for the last sample listed, the maximum swelling of these erythrocytes was between 128 and 156 per cent of their initial volume. The last two samples listed, from the patient W M , were drawn respectively before and two weeks after splenectomy. A great improvement in the patient's general condition was observed after splenectomy. The postoperative blood sample showed lessened red-cell fragility as indicated by the point of beginning hemolysis and by greater maximum swelling, compared with that of the sample drawn before operation. In all instances the maximum swelling was predicted fairly closely from the mean surface area of the cells, and the *R*-values were about the same (within limits of experimental error) as those of normal erythrocytes at all tonicities up to the point of maximal swelling.

*Hypochromic anemia* In studies on patients with varying degrees of anemia presumed to be due to simple iron deficiency, different patterns of osmotic behavior of the red cells were found among bloods showing varying degrees of hypochromia and microcytosis. The first example in this group, cited in table 3 and figure 3, was a blood sample from a Negro woman, F D , with mild anemia, with hemoglobin concentrations in the whole blood 9.9 grams and in the packed cells 30 grams per 100 cc , and the mean cell volume 75 cubic microns. The unusually high value for maximal swelling of these cells, 199 per cent of their initial volume, agreed closely with the predicted value and the swelling followed very closely the expected osmometric swelling up to the maximal point, with an average *R*-value 1.00. In the last three examples cited in this group, R G , D P , L W , with cells showing a greater degree of microcytosis and hypochromia, the swelling of the cells followed the expected osmometric volumes up to a certain point, in each case after the beginning of hemolysis, but thereafter the swelling of the remaining cells was less than expected (*R*-values at first 1.00, then 0.80 to 0.91) and the maximum volumes attained were less than the values predicted from the mean surface area.

*Thalassemia* The first of the two sets of data listed under thalassemia in table 4 and illustrated in figure 4 concern the erythrocytes of a 15 year old boy, G B , who exhibited typical manifestations of Mediterranean or Cooley's anemia, with large spleen, moderate skeletal changes, moderate anemia and normoblastemia. The red cells were moderately large in mean volume and diameter, but thinner than normal. There was the usual wide span of osmotic resistance, with hemolysis beginning at

0.5 and not complete until around 0.1 per cent NaCl. At all tonicities the observed swelling was less than the expected osmometric swelling, with the R-value gradually diminishing, from 0.88 to 0.81, but the final maximal volume of 213 per cent approached closely the value predicted from the mean surface area. The second set of data listed in the table relate to this boy's brother, J. B., who showed no physical manifestations of this disorder but whose blood showed the typical target cells and microcytosis such as have been noted by several investigators<sup>25b</sup> among essentially healthy members of the families of patients suffering with Cooley's disease. These cells were small and thin, they too had a rather wide span of resistance, but their swelling followed closely the normal expected pattern up to a maximum volume of 199 per cent, with behavior like that observed in the case of F. D., illustrated in figure 3, with mild hypochromic microcytic anemia. Two other siblings in this family had mild anemia that did not respond to the administration of iron, with microcytic hypochromic erythrocytes and target cells like those of the blood of J. B. and displaying similar osmotic behavior. Apparently the defect responsible for poor osmometric behavior in the cells of the patient, G. B., was not present in the cells of the siblings, although the microcytes and target cells in such families have been regarded as part of the genetic picture of thalassemia.

*Sickleemia* The two patients, M. J. W. and M. W., represented in figures 5 and 6, respectively, displayed noteworthy differences in symptomatology as well as differences in the osmotic behavior of their red cells. The first, M. J. W., represented in figure 5, had experienced little illness, was not known to have had crises of anemia or jaundice at any time, and the spleen was not palpable. Sickling of his cells in a sealed cover glass preparation occurred quickly and involved practically 100 per cent of the cells. The other, represented in figure 6, had suffered repeated crises of abdominal pain associated with anemia, slight jaundice, and enlargement of the spleen. Sickling in his blood occurred rather slowly and involved about half of the cells in a sealed cover glass preparation allowed to stand twenty-four hours at room temperature. Reticulocytes in the two bloods were respectively 2.6 and 11.2 per cent. The erythrocytes of both bloods were thinner than normal (see data in table 3). The swelling of the cells in both bloods at all tonicities was considerably less than the expected osmometric swelling. The cells represented in figure 5 attained a maximum volume close to that predicted from the surface area, but in figure 6 the observed maximal volume is seen falling far below the predicted value. Figure 5 shows a considerably increased osmotic resistance, with only slight hemolysis appearing at tonicities from 0.45 to 0.35 per cent NaCl and complete hemolysis at around 0.125 per cent. Figure 6 shows a diminished resistance of part of the cells, with hemolysis beginning in 0.6 per cent NaCl solution and complete at around 0.225 per cent. Data on the red cells of a third patient (subject E. J.) with sickleemia cited in table 3 resemble those of the subject M. J. W. in that the R-values were consistently low but the observed maximal volume agreed closely with the predicted maximal volume.

*Pernicious anemia* Observations on the erythrocytes of a patient, J. W., suffering from pernicious anemia are illustrated in figure 7, other data on these cells are

listed in table 3. This sample of blood was obtained when the reticulocyte count was 25 per cent, following the administration of liver extract. The erythrocytes were typically large in all dimensions, their swelling at all tonicities was less than the expected osmometric swelling, and the maximal volume found was considerably less than predicted from the mean surface area. These observations are presented not to suggest that such findings are necessarily typical of pernicious anemia (further observations on other patients are lacking) but to illustrate again a pattern of abnormal behavior found in red cells of pathologic type.

*Miscellaneous.* Normal osmotic behavior of erythrocytes, corresponding closely to that illustrated in figure 1, has been found in cases of polycythemia vera, cirrhosis of the liver, anemia associated with acute and chronic nephritis, liponephrosis and other metabolic diseases.

### DISCUSSION

These observations indicate that normal human erythrocytes, and also some abnormal types of red cells varying considerably in size, shape and hemoglobin content, display a remarkably uniform pattern of osmotic behavior in hypotonic salt solutions, swelling like perfect osmometers up to a maximum volume of spheres within the limits of their surface areas. Contrasting pictures may be seen in studies of the thick cells, spherocytes, of congenital hemolytic icterus and the thin cells of mild hypochromic anemia as illustrated in figures 2 and 3 respectively. The patterns of behavior exhibited by the two types of cells appear to be governed by fundamentally similar principles, although their relative "fragility" appears to be quite different. The abnormal fragility of the cells of congenital hemolytic icterus probably depends on the character of the internal structure of the cell that is responsible for their greater than normal thickness rather than on any abnormality of the surface membrane affecting its permeability or susceptibility to stress.

The well known increased resistance, or decreased fragility, of the red cells of thalassemia, indicated by the points of beginning and complete hemolysis in the Hamburger series, can be explained by two mechanisms demonstrated in figure 4. First, the cells behave as poor osmometers, swelling less than expected at all tonicities, and second, the very thin cells of large diameter are capable of much greater percentile swelling than normal cells within the limits of their surface area. Such explanation applies also to the somewhat increased osmotic resistance of the cells of the patient with sickle cell anemia, represented in figure 5.

The red cells in all three cases of sickle cell anemia cited in table 3 behaved as poor osmometers, swelling less than expected at all tonicities. The span of hemolysis was wide in each instance. In two cases the observed maximal swelling of the cells agreed closely with the value predicted from the mean surface area, but in the case of M. W. (fig. 6), the observed maximal swelling was much less than predicted. It seems possible that the patients represent distinctly different clinical types, one showing merely the sickling trait with increased resistance of the cells, and the other a more complicated picture of a hemolytic type of anemia with increased fragility of at least a part of the red cells. The patient M. W. displayed

symptoms and physical manifestations, with crises of jaundice, anemia, and enlargement of the spleen, compatible with this concept. There is of course the possibility that a hemolytic trait may be superimposed on the sickling trait.

Investigators who have found erythrocytes swelling less in hypotonic fluids than should be expected if they imbibe water like perfect osmometers have offered several explanations for this behavior: namely, (1) that salts escape from the cells in the course of their swelling, not necessarily at a constant rate nor reaching the same equilibria at different tonicities, (2) that variations in the water content of the cells may be large, so that the formula for osmometric swelling, based on an assumed initial water content of 70 per cent, cannot be applied to all erythrocytes alike, (3) that some of the water in the cells is "bound" to hemoglobin and other constituents and is not transferrable by osmosis, (4) that phenomena of gelation in some cells may lead to physical conditions quite unlike those assumed for a simple sac or envelope containing hemoglobin and other osmotically active substances in solution.<sup>19, 21</sup>

It would appear that any one or several of these explanations might apply to the osmotic behavior of abnormal types of erythrocytes such as are cited here. It is well to note, with regard to seemingly contradictory statements of various investigators regarding the osmotic behavior of "the erythrocyte," that generalizations are not sound when they imply (as some have) that observations on the red cells of one or a few members of a species can be applied to all individuals of the species or to other species. It must be borne in mind that erythrocytes of different species differ considerably in their characteristics of osmotic fragility, also, that differences in osmotic behavior of red cells drawn from the same individual at different times may arise from alterations in cellular composition and metabolic activities induced by various pathologic conditions, which may not be readily apparent and may even be transient.\*

#### SUMMARY

Normal human erythrocytes suspended in the series of hypotonic salt solutions employed for testing red cell fragility behave like nearly perfect osmometers throughout the series, with their maximum swelling, usually around 175 per cent of their initial volume, sharply defined by their mean surface area. The same principles govern the swelling and hemolysis of some abnormal types of erythrocytes with different characteristics of abnormal fragility or resistance to osmotic hemolysis. The red cells of congenital hemolytic icterus exhibit essentially normal osmotic behavior, but since the spherocytes can swell very little within the limits of their surface area they rupture at higher tonicities, with the maximal swelling in most instances around 150 per cent of their initial volume. Thin cells are capable of greater swelling than normal cells. Moderately hypochromic erythrocytes from patients with mild anemia behaved like perfect osmometers throughout the series,

\* The effects of environmental factors, e g, anticoagulants, diluting fluids, time of preservation, temperature, etc., on erythrocytes prior to studies of their osmotic behavior have been thoroughly discussed in several papers of the symposium on blood preservation recently published in the *J. Clin. Investigation*, 26:591-755, July 1947.



to attain a maximal swelling of around 200 per cent, this value agreeing with predictions based on their mean surface area

Erythrocytes of patients with thalassemia, sickle cell anemia and pernicious anemia exhibited less than the expected osmometric swelling throughout the series of hypotonic solutions. The cells behaving as imperfect osmometers displayed varying patterns of hemolysis, and of maximal swelling in relation to predicted values. The increased osmotic resistance characteristic of the cells of thalassemia is accounted for by two mechanisms: they swell less than normal cells at each tonicity, and, being thin, they undergo greater swelling (around 220 per cent) before they are hemolyzed.

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# A METHOD FOR DETERMINING THE FORM OF THE DISTRIBUTION OF RED CELL RESISTANCES TO SIMPLE HEMOLYSINS

By ERIC PONDER, M D , D Sc

THE DETERMINATION of the way in which the resistances of red cells are distributed with respect to hypotonicity (the erythrocyte fragility test) is a familiar laboratory procedure, and methods have been described for finding the distribution of the resistances of red cells to heat,<sup>1 2</sup> to acid,<sup>3</sup> to shaking,<sup>4 5 6</sup> to saponin hemolysis<sup>7 8</sup> and to hemolysis by lysolecithin.<sup>9</sup> It is now recognized that the form of the frequency distribution of red cell resistances to hypotonicity is determined primarily by the variations in the shape of the cells of the system,<sup>10--</sup> and it is reasonable to think that the forms of the frequency distributions of resistances to lysins such as saponin or digitonin depend on the chemical nature and spatial arrangement of the components of the red cell architecture, i.e., on the "consist"\* of the cell or of its surface ultrastructure. Just as it is necessary to have a method by means of which fragility can be described quantitatively before its relation to shape can be appreciated, so it is necessary to have a method by means of which the distribution of resistances to lysins can be described quantitatively before it is possible to study the relation of the resistance to chemical composition, spatial arrangement, etc.

The purpose of this paper is to describe a method for measuring the resistance of red cells to simple hemolysins, to define the range of normal variation, and to give a few illustrations of abnormal variations in resistance which occur under conditions in which it is reasonable to believe that abnormal variations in the consist of the members of the cell population have occurred. Emphasis will be placed on the observations being made and treated so that the maximum amount of information can be extracted from them, because the full potentialities of methods for determining red cell resistances are not realized when they are carried out in simplified form. Further, it should be remarked at the outset that the method to be described is of general application, although it is illustrated by results obtained with only two simple hemolysins, in addition to the results of fragility determinations.

## I COMPOSITION OF THE HEMOLYTIC SYSTEMS

To illustrate the procedure, the two lysins saponin and digitonin have been selected for several reasons. More is known about the kinetics of lysis by saponin than by any other lysin, and the flat form of the frequency distribution of resistances makes it particularly useful for picking up bimodalities, etc. Digitonin is selected because it is one of the few powerful lysins which can be obtained in the pure state (unlike the saponins, the activity of which is variable), and free from inhibitory contaminants (unlike most preparations of the bile salts).

(a) *Saponin* Add 10 mg of quillia saponin (British Drug Houses) to 100 ml of 1 per cent NaCl,

From The Nassau Hospital, Mineola, N Y

\* This is a noun, the general use of which has been urged by Schlegel<sup>17</sup>, it is employed in describing the composition of railway trains. Its definition is "Consist," "The singular elements of which something is constituted together with all the relevant spatial arrangements of these elements." As Schlegel points out, there is no other word in our language which conveys quite the same idea.

this gives 1 in 10,000, or a 200γ/2 ml solution of the lysin. A series of dilutions is made from this, so that 100 ml volumes of a series of solutions of saponin containing 100, 80, 60, 50, 40, 30, 25, 20, 15, 12, 10, 8, 6, 4, 3, 2, and 1 γ per 2 ml are obtained. The solutions are stored in stoppered bottles at temperature 4 C, and keep for about 1 month.

(b) *Digitonin* 100 ml volumes of solutions of digitonin (Merck) in 1 per cent NaCl, and containing 20, 17.5, 15, 12.5, 10, 8.75, 7.5, 6.25, 5.0, 3.75, 2.5, and 1.25 γ per 2 ml are prepared. Because of the steepness of the digitonin percentage hemolysis curve, intermediate concentrations, e.g., 8.125 γ/2 ml, may be needed, if so, 2 ml of such a concentration can be made by mixing 1 ml of the concentrations above it and below it in the series, i.e., 8.75 γ/2 ml and 7.5 γ/2 ml. As the final values for hemolysis are reached within fifteen to thirty minutes in systems containing digitonin, the need for making additional observations at such intermediate concentrations can be appreciated soon after the experiment is set up, systems containing the intermediate concentrations can then be added if necessary, and the results can still be read at the end of 5 hours without any error having been introduced. The digitonin solutions keep at 4 C for about 1 month.

(c) *Hypotonic NaCl* A series of solutions of hypotonic NaCl is prepared,\* starting with a 0.5 per cent NaCl and descending by a common difference of 0.02 units, i.e., 0.50, 0.48, 0.46, ... per cent NaCl, in the scale of tonicity (the tonicity of a 1 per cent NaCl corresponds to 1 tonicity unit). The lowest member of the series should be 0.14 per cent NaCl. These solutions are stored at 4 C. When 0.5 ml of a red cell suspension, with a tonicity of 1.0, is added to 2 ml of each member of the series, we get a new series for the final tonicity  $T_1$  of the mixture, this is

$$T_1 = \frac{2T_0 + 0.5}{2.5}$$

where  $T_0$  is the tonicity of the member of the first series. The common difference on the new series for the final tonicity is 0.016, so that the new series runs 0.60, 0.584, 0.568, ... with a lowest member of 0.312 per cent NaCl or tonicity units. For convenience in differentiating the experimental curves, the members of the new series can be described by integers starting with 0.60 = 0, so that 0.584 = 1, 0.568 = 2, 0.312 = 18, as in the subsidiary scale on the abscissa of the curve marked T in figure 2.

(d) *Cell suspensions* The volume concentration  $\rho$  of the red cells in freshly drawn heparinized blood is found with a high speed hematocrit, and the cells of 2.5 (0.4/ $\rho$ ) ml of blood, after being washed three times with 1 per cent NaCl, are finally suspended in 25 ml of NaCl-buffer at pH 7.0 †

(e) *The hemolytic systems* The hemolytic systems, contained in three series of 100 × 13 mm tubes, are prepared by adding 0.5 ml of the red cell suspension to 2 ml volumes of the various concentrations of saponin, digitonin, and hypotonic NaCl. The lysins are put into the tubes first, and allowed to reach the temperature at which the determinations are to be made, 0.5 ml of suspension is then added to each tube, with immediate shaking to produce mixing. Any desired temperature can be obtained by using water

\* The NaCl used must be silver-free,<sup>18</sup> as most C.P. preparations now are. It must also be dry. Commercial preparations may contain as much as 10 per cent of water after standing around the laboratory, they should be dried for twenty-four hours at 120 C before use.

† The NaCl-buffer is made by mixing 75 ml of 1.2 Gm/100 ml NaCl with 25 ml of a mixed buffer composed of 72 ml of M/15  $\text{Na}_2\text{HPO}_4$  and 28 ml of M/15  $\text{NaH}_2\text{PO}_4$ . The depression of freezing point of this NaCl-buffer is the same as that of 1 Gm/100 ml NaCl (i.e., its tonicity is 1.0), and its pH is 7.0 at 25 C.

The pH of the systems requires to be controlled at a known value because the activity of many lysins, e.g., saponin and the bile salts, has a marked pH dependence. It is usual to make measurements of fragility in systems containing unbuffered hypotonic NaCl on the grounds that the situation may be complicated by the addition of the ions in the buffer mixtures. This is true, but the situation is also liable to be complicated by uncontrolled variations in pH, especially if the red cell suspension is dilute.<sup>19</sup> If the suspension is more concentrated, the effects may be very much less,<sup>20</sup> but, since a complete investigation of the distributions of resistances to simple lysins and to hypotonicity would include measurements of resistances at a variety of pHs, one may as well buffer the systems from the start.

baths, but 25 C is a convenient temperature which can easily be maintained to within  $\pm 1$  C for five hours at a time in the average laboratory room

The hemolytic systems are mixed, either by rotary shaking or by inversion, every fifteen minutes, and the amount of hemolysis in each is determined at the end of five hours

## 2 MEASUREMENT OF PERCENTAGE HEMOLYSIS

To find the percentage of complete hemolysis present in any one of the hemolytic systems at the end of five hours at 25 C, the cells are thrown down by spinning for a few minutes at 2000 r p m. The supernatant fluid can then be poured off into a dry vial without disturbing the cells. Two ml of this supernatant fluid is added to 10 ml of distilled water, and the optical density is measured at a wave length of about 4500 Å (blue filter) with a Lumetron photometer or with any other photometer of a similar type. This measurement is converted into a value for percentage

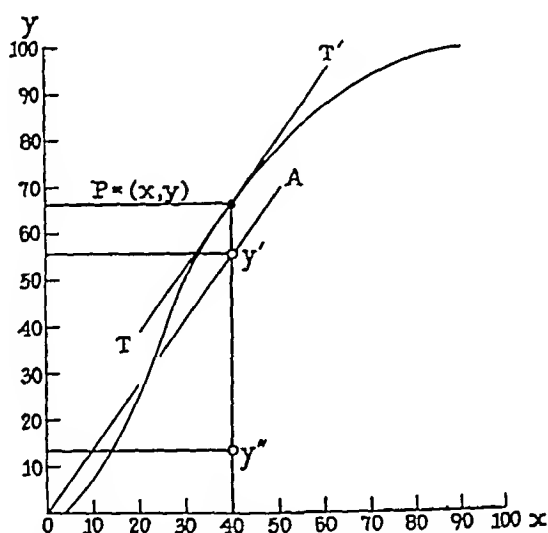


FIG. 1. DIAGRAM TO ILLUSTRATE THE METHOD OF GRAPHIC DIFFERENTIATION  
For description, see text

hemolysis by referring to a calibration curve, prepared by measuring the optical densities corresponding to 100, 50, 25, and 12.5 per cent of complete hemolysis of the cells contained in the hemolytic systems. When measured at 4500 Å, the optical density is so related to the concentration of Hb present that percentages of complete hemolysis are found with about the same degree of accuracy over the whole percentage hemolysis range.

A separate calibration curve must be prepared for each suspension used, in practice, one uses the pooled contents of several completely hemolyzed systems for obtaining the 100 per cent point, and finds the other points by successive dilutions by powers of 2.

## 3 PLOTTING AND DIFFERENTIATION OF THE CURVES

The values of percentage hemolysis  $P$  are plotted against the quantity of lysin in  $\gamma$  contained in the system, using the same scales for ordinates and abscissae as those used in figures 2, 3, and 4. The choice of the scales is important, as it de

termines the ease and accuracy with which differentiation can be carried out. Smooth sigmoid curves are drawn through the experimental points, a possible error of about  $\pm 2$  per cent being allowed in the case of each. If inspection shows that the curve is bimodal or polymodal, the procedure is still to draw the smoothest curve through the experimental points. It will be obvious that the more points

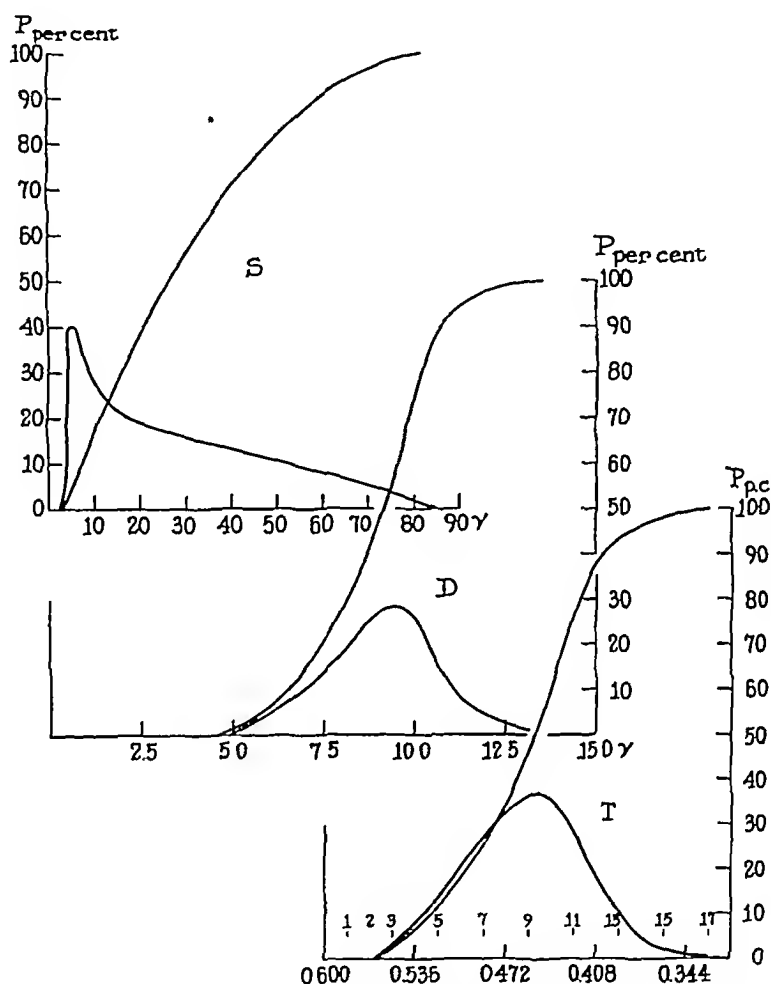


FIG. 2. NORMAL FREQUENCY DISTRIBUTIONS OF RESISTANCES, TOGETHER WITH THEIR INTEGRALS (THE EXPERIMENTAL CURVES)

Ordinates, percentage of complete hemolysis, abscissae, quantity of lysin in system in  $\gamma$  except in curve T, where the abscissa shows the tonicity in tonicity units. Subsidiary scale on abscissa of curve T is that used in differentiating. Curve S, results for saponin, curve D, results for digitonin, curve T, results for hypotonic NaCl. Reduction,  $2\times$ .

there are, the more certainly can the course of the curve be determined, and in some cases of suspected polymodality it may be necessary to repeat the entire set of determinations with the addition of new and strategically placed concentrations of the lysin.

The sigmoid curve is now differentiated graphically by using the following principle. If  $TT'$  is the tangent at a point P with coordinates  $x$  and  $y$  (fig. 1), and if  $OA$ , the parallel through the origin, cuts the ordinate of P at  $y'$ , the value of

$dy/dx$  at the point P is  $y'' = y'/x$ . Proceeding along the sigmoid curve systematically, tangents and their parallels through the origin are drawn with the aid of rulers fixed so as to move in parallel, these can be purchased from any dealer in draughtsman's materials. The value of  $y'$  (read off on the ordinate) at which the parallel through the origin cuts the ordinate of the point under consideration is noted and is divided by the value for the x-coordinate of the point (read off on the

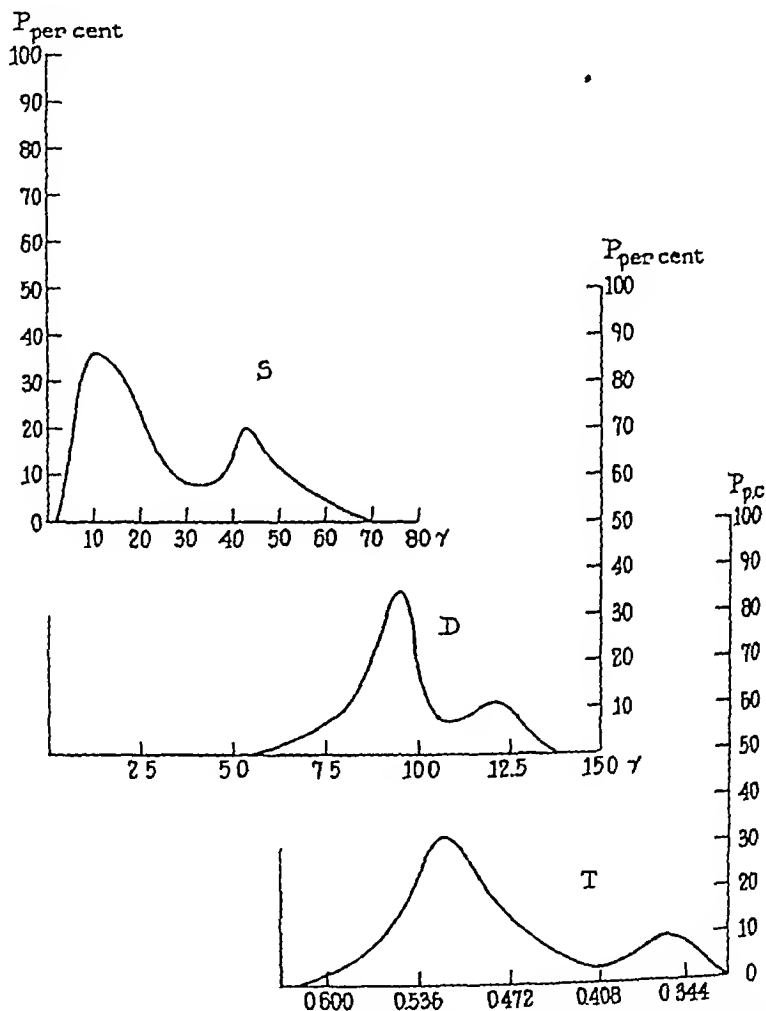


FIG 3 FREQUENCY DISTRIBUTIONS AND THEIR INTEGRALS FOLLOWING MASSIVE HEMORRHAGE

Ordinates, abscissae, etc., as in fig 1

abscissa), the result of the division is a value of  $y''$ , the ordinate of the corresponding point on the differential curve. To change the scale on which the differential curve is plotted, the value of  $y'$  can be divided, not by  $x$ , but by  $x/s$ , where  $s$  is a convenient scaling constant, the values  $s = 10$  for saponin,  $s = 1$  for digitonin, and  $s = 2$  for hypotonic NaCl are convenient because the frequency distributions are then plotted on such a scale that their shape and variations in it can be easily appreciated by eye. These values have been used in constructing the differential curves in figures 2, 3, and 4. If  $s = 1$  were used for saponin, for example, the differential curve would be so flat that variations in its shape would not be apparent on simple inspection.

This process of differentiation can be carried out quickly and easily, and is all done on the same piece of graph paper. The number of tangents which require to be drawn depends to some extent on the shape of the sigmoid curve, but one should be drawn at each experimental point for a start. The process is completed by drawing a smooth curve through the points  $y''$  obtained by differentiation. In each case, the result is a frequency curve showing the distribution of red cell resistances to the

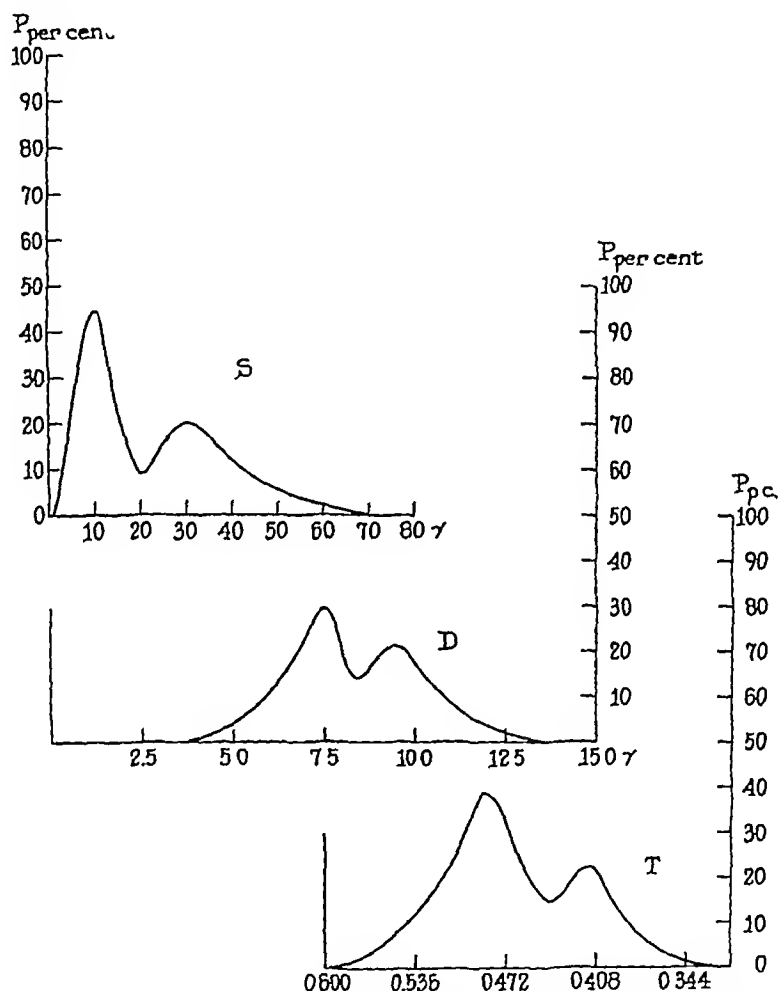


FIG. 4. FREQUENCY DISTRIBUTIONS AND THEIR INTEGRALS FOLLOWING A HEMOLYTIC EPISODE

Ordinates, abscissae, etc., as in fig. 1

lysin, on an abscissa showing the amount of lysin in the system in  $\gamma$  (or the tonicity of the system in tonicity units in the case of hypotonic NaCl)

#### 4. NORMAL VALUES

To explore the range of normal variation, the frequency distributions of resistances to saponin, digitonin, and hypotonic NaCl have been obtained at 25 C for the red cells of 12 normal blood donors. Table 1 shows the average, lowest, and highest values found for various statistics of the curves. The interdecile difference between the lowest and highest deciles,  $D_1$  and  $D_2$ , has been used as a measure of



scatter, and the skewness has been calculated from the deciles  $D_1$  and  $D_2$  and the median  $M$ , as

$$\frac{(D_2 - M) - (M - D_1)}{(D_2 - M) + (M - D_1)}$$

by analogy with Bowley's measure of skewness. The three distribution integrals with average values are plotted, together with the distributions obtained from them by differentiation, in figure 2.

The distribution of resistances to saponin is normally negatively skew, with a long tail spreading out towards its upper extreme. The resistance distribution to digitonin is usually positively skew, and its scatter is relatively small. The normal

TABLE 1

	Average	Lowest	Highest
Saponin			
Median	26.5	22.0	28.5
Lower extreme	3.5	3.0	4.5
Upper extreme	85.0	73.0	96.0
Inter-decile diff	52.5	44.0	57.5
Skewness	-0.30	-0.15	-0.41
Digitonin			
Median	9.20	9.05	9.45
Lower extreme	4.70	3.80	5.35
Upper extreme	13.5	12.5	14.9
Inter-decile diff	4.25	4.05	4.50
Skewness	0.29	0.05	0.36
Hypotonicity			
Median	0.448	0.424	0.472
Lower extreme	0.566	0.584	0.552
Upper extreme	0.328	0.360	0.312
Inter-decile diff	0.131	0.103	0.139
Skewness	0.14	-0.06	0.22

distribution of resistances to hypotonic NaCl at pH 7.0 is usually symmetrical, although it may have a small skewness in either direction. None of the observed distributions shows any great individual variation from the average distribution obtained for normal red cells.

## 5. DISCUSSION, WITH EXAMPLES

The frequency distributions obtained experimentally with each lysin represent the distribution of the resistances of the  $N$  cells in the general circulation at the time the blood is drawn. This number is maintained at a constant level in the normal individual by the addition of new cells at a rate  $P$  and by the removal of old cells at a rate  $Q$ , so that  $dN/dt = P - Q$ . Changes in the rates of production and destruction result in changes in the value of  $N$  accompanied by *transient* changes in the form of

the resistance distribution, variations in the distribution of the resistances of the cell in the population  $N$  can, however, be brought about in another way. The  $P$  cells which are added to the population  $N$  in each unit of time themselves constitute a population with resistances distributed according to some form of frequency distribution, the same applies to the  $Q$  cells which are removed from the population  $N$  in unit time, and it can be shown that in the steady state the distribution of resistances in the population  $P$  is the same as that in the population  $Q$ , that the form of these distributions determines the form of the distribution in the population  $N$ , and that, if the life of the red cell in the circulation depends on its resistance to the lysin in question, the  $P$  and  $Q$  distributions must be more negatively skewed than the  $N$  distribution.<sup>15</sup> It follows that *persistent* changes in the form of the distribution of resistances in the population  $N$  are reflections of persistent changes in the form of the distribution of resistances in the populations  $P$  and  $Q$ , and that *transient* changes in the distribution of resistances in the population  $N$  are due to sudden changes in the rate at which new cells are added to it, or in the form of the resistance distribution in the population  $P$ . Sudden changes in the rate of removal of old cells or in the distribution of resistances in the population  $Q$  can conceivably produce similar transient changes in the population  $N$ .

Hemorrhagic or hemolytic episodes supply the clearest instances of transient changes in the form of the distribution of resistances to saponin, digitonin, and hypotonic NaCl, as is illustrated by the following two examples.

*Example 1* Hemorrhage from duodenal ulcer seven days before. Red cells 2.2 millions, Hb 5.7 Gm, reticulocytes 6 per cent. All three resistance distributions bimodal. Saponin distribution has modes at 107 and 437, digitonin distribution has modes at 9.57 and 127, hypotonic NaCl distribution has modes at 0.520 and 0.360 tonicity units (fig. 3).

*Example 2* Hemolytic anemia accompanying metastatic carcinoma with pylorus as the primary site. Red cells, 1.8 millions, Hb 4.6 Gm, reticulocytes 13 per cent. All distributions bimodal. Saponin modes, 107 and 307, digitonin modes, 7.57 and 9.47, hypotonic NaCl modes, 0.488 and 0.416 tonicity units (fig. 4).

In these cases, the bimodalities are presumably due to a new population with its own frequency distribution of resistances having been added to the existing one at the time of the hemorrhagic or major hemolytic episode. The existing data do not allow the situation to be analyzed further. It would be almost certainly wrong, for example, to identify one of the modes as being produced by the reticulocytes in the population. All that can be said is that *at least* two distinguishable frequency distributions are superimposed.

Illustrations of persistent abnormalities in the form of one or more of the frequency distributions are less striking and not so easy to supply. Apart from the marked change in the distribution to saponin found in pernicious anemia in relapse<sup>16</sup> and to lysolecithin found in congenital hemolytic icterus,<sup>9</sup> the abnormalities are usually encountered during routine determinations of the resistance distributions in hypoplastic and toxic anemias, and in the anemias associated with leukemia, the various forms of lymphoma, etc. The italicized values found in the following three cases are outside the normal range of variation as given in Table 1.

*Example 3* Hodgkin's disease Red cells, 3.9 millions, Hb 10.4 Gm Saponin distribution median 187, lower extreme, 27, upper extreme, 1007, skewness,  $-0.60$  Digitonin distribution median, 171, lower extreme, 367, upper extreme, 1257 Hypotonic NaCl distribution median 0.456, lower extreme, 0.636, upper extreme, 0.294, interdecile difference, 0.233

*Example 4* Aplastic anemia Red cells, 1.9 million, Hb, 5.2 Gm Saponin distribution median, 271, lower extreme 47, upper extreme, 567, interdecile difference, 407, skewness,  $-0.35$  Digitonin distribution median, 917, skewness 0.45 Hypotonic NaCl distribution, no abnormalities

*Example 5* Normocytic anemia due to chronic infection Red cells, 3.8 millions, Hb, 9.2 Gm Saponin distribution median, 317, lower extreme, 27, upper extreme, 637, skewness,  $-0.03$  Distributions to digitonin and to hypotonic NaCl substantially normal

Much more data will have to be accumulated before it is possible to classify these persistent abnormalities in the form of the frequency distributions to lysins. It is significant that all those which I have observed have been associated with anemia. In the meantime the persistent abnormalities can be looked upon as having much the same significance that abnormal Price-Jones curves would have, i.e., as showing that the normal red cell population has been partially or wholly replaced by populations having different resistance characteristics.

### SUMMARY

A method is described for determining quantitatively the form of the resistance distributions of red cell resistances to simple hemolysins. The normal range of variation is given for the resistance distributions to saponin, digitonin, and hypotonic NaCl, all at pH 7.0, and examples of departures from the normal, which may take the form of bimodalities after hemorrhagic or hemolytic episodes or of more persistent changes in the characteristics of the distributions, are provided.

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# THE HEMOGLOBIN OF HEALTHY COLLEGE UNDERGRADUATES AND COMPARISONS WITH VARIOUS MEDICAL, SOCIAL, PHYSIOLOGIC AND OTHER FACTORS

By CLARK W. HEATH, M.D.

AN UNUSUAL opportunity for obtaining data concerning the blood of healthy young college men has been afforded in the work of the Grant Study and the Harvard Fatigue Laboratory. The subjects were Harvard undergraduates, selected for good health and adjustments.\* In addition to medical examination and blood examination, these men were studied by a variety of technics including physiologic tests, anthropologic measurements and psychologic and social surveys. The average age was 19 years, 7 months, the range 17 years to 25 years. Sixty-one per cent were born in Northeastern United States. The remainder were born elsewhere in the United States with the exception of three foreign-born. Table 1 indicates the distribution of hemoglobin findings and red blood cell counts in 153-259 of these young men.

## METHODS

Sahli hemoglobin determinations and red blood cell counts were obtained on oxalated venous blood in the course of the physical examination. Sahli tubes and solid glass standard were calibrated from bloods whose oxygen capacity had been ascertained, converting to grams of hemoglobin by the factor 0.746. Oxygen capacities were determined by the Van Slyke method in the Fatigue Laboratory on heparinized arterial blood, taken as a rule in the fasting state. The two determinations were made at varying times apart, usually a few days or weeks.

The means of hemoglobins are only slightly less, as a rule, than those reported elsewhere in this country, but higher than those reported from England.† The wide variation of hemoglobins is worthy of comment. In the total series of Grant Study participants only one case was omitted in which rather low hemoglobin seemed possibly the effect of a chronic infection. In other instances of lower

From the Grant Study, Department of Hygiene, the Fatigue Laboratory, Harvard University, the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

\* Detailed description and method of selection of the men are given in reference 1.

† M. M. Wintrobe,<sup>2</sup> 86 male medical students, Newcomer Method, Mean 16.0, S.D. 0.9.

MacFarlane and O'Brien,<sup>3</sup> 29 medical students and members of hospital staff, Oxygen Capacity Method, Mean 15.9.

E. E. Osgood,<sup>4</sup> 137 medical students, Osgood Method, Mean 15.76, S.D. 1.09.

Andresen and Mugrage,<sup>5</sup> 36 men of various ages, Van Slyke Method, Mean 16.58, S.D. 0.82.

C. Price-Jones,<sup>6</sup> 100 men (London) of various ages, Haldane Method, Mean 14.55, S.D. 0.54.

C. Price-Jones, Vaughan and Goddard,<sup>7</sup> 90 men, various ages, Haldane Method, Mean 14.55, S.D. 0.69.

R. L. Haden,<sup>8</sup> 20 men ages 18 to 30, Van Slyke Method, Mean 15.83, S.D. 0.76.

P. C. Foster and Johnson,<sup>9</sup> 115 male students, Van Slyke Method, Mean 15.63, S.D. 0.92.

C. J. Hamre and Au,<sup>10</sup> 137 university students (Honolulu) (varying racial groups), Acid Hematin and Van Slyke Methods, Mean 15.10, S.D. 1.11.

C. F. Nelson and Stoker,<sup>11</sup> 350 men of various ages, Van Slyke Method, Mean 15.03, S.D. 1.15.

hemoglobin levels, although determinations were repeated, only the first determination is reported here. Cases in which the hemoglobin level was at the extremes, as 13.5 grams or less, and 16.0 grams or more, gave no clinical evidence to explain such levels. In one young man the initial Sahli hemoglobin was 13.1 Gm, one week later, 13.4 Gm, six weeks after that, 14.35 Gm, finally, four days after the third determination his hemoglobin was 14.6 by the Van Slyke method. Another subject was observed to have a hemoglobin level of 16.1 Gm. Two years previously, determinations at Thorndike Memorial Laboratory were 15.8 Gm and 17.2 Gm, two weeks apart. One month after the Sahli determination at the Grant

TABLE I—*Distribution of Hemoglobin Determinations and Red Blood Cell Counts*

Class Interval	Hemoglobin by Sahli Test		Hemoglobin by O <sub>2</sub> Capacity		Class Interval R B C	Red Blood Cells	
	No. Cases	%	No. Cases	%		No. Cases	%
<i>Gm Hgb</i>					<i>mils /cu mm</i>		
12.6-12.9	1	0.4	1	0.7	4.20-4.29	1	0.4
13.0-13.3	1	0.4	1	0.7	4.30-4.39	3	1.2
13.4-13.7	8	3.1	8	5.2	4.40-4.49	1	0.4
13.8-14.1	18	7.0	10	6.5	4.50-4.59	1	0.4
14.2-14.5	36	13.9	26	17.0	4.60-4.69	8	3.1
14.6-14.9	27	10.5	25	16.3	4.70-4.79	16	6.2
15.0-15.3	53	20.1	25	16.3	4.80-4.89	24	9.3
15.4-15.7	43	16.7	26	17.0	4.90-4.99	37	14.3
15.8-16.1	50	19.4	16	10.5	5.00-5.09	71	27.4
16.2-16.5	19	7.4	9	5.9	5.10-5.19	50	19.3
16.6-16.9	2	0.8	2	1.3	5.20-5.29	31	12.0
17.0-17.3	0	0.0	3	2.0	5.30-5.39	11	4.2
17.4-17.7	0	0.0	0	0.0	5.40-5.49	4	1.5
17.8-18.1	0	0.0	1	0.7	5.50-5.59	1	0.4
Totals	258	99.7	153	100.1		259	100.1
Range	12.6-16.8 Gm		12.6-17.8 Gm			4.25-5.56 Mils	
Mean	15.19 Gm		15.13 Gm			4.98 Mils	
St dev	.75 Gm		.86 Gm			.13 Mils	
Coeff var	4.9%		5.6%			2.6%	

Study, the hemoglobin by the Van Slyke method was only 14.6 Gm. With due consideration for the variations that may take place in the hemoglobin level of an individual from day to day, as well as the possible errors in determination,\* it appears that individual persons have hemoglobin ranges that are native to them.

\* Even under carefully standardized conditions, the errors in performing the Sahli test upon the same blood sample may be considerable. In one series of observations, when the test was repeated by the same observer, only 13 per cent of the readings were identical, 77 per cent were within 2 per cent, or 0.31 Gm, 95 per cent within 3 per cent, or 0.47 Gm. When the test was repeated by different individuals, 19 per cent of the readings were identical, only 32 per cent were within 2 per cent, or 0.31 Gm, only 44 per cent within 3 per cent, or 0.47 Gm. (Figures furnished by G. A. Daland, Thorndike Memorial Laboratory.)

and that are compatible with good function of their particular physiology. It is hardly necessary to detail the wide variation of other measurements in any population, such as height, weight, basal metabolism and pulse rate, variations not necessarily due to health, nutrition or other environmental influences.

An attempt has been made to search for factors which might correlate with higher or lower hemoglobins in these 258 men, selecting from the extensive data available in the Grant Study. As will be seen, the search was largely unavailing. Only in the case of sitting pulse rate taken at the time of medical examination and blood collection was there a statistically significant relationship with hemoglobin level. Other factors showed certain trends as will be indicated.

The method of relating hemoglobin level to various factors is illustrated by table 2. The Chi-square ( $\chi^2$ ) test,<sup>12</sup> which compares observed results with those expected by chance, was employed. Table 2 shows that there is an excess of individuals having lower hemoglobins who have slower pulse rates, and a deficiency of individuals with lower hemoglobins having faster pulse rates. (The average

TABLE 2.—*Comparison of Hemoglobin and Pulse Rate (Sitting, at time of Medical Examination)*

Pulse Rate	Hemoglobin					
	15.1 Gm /100 cc. or less		15.2 Gm /100 cc. or more		Totals	
	No	%	No	%	No	%
75 Beats per min. or less	77	65.8	64	50.4	141	57.8
76 Beats per min. or more	40	34.2	63	49.6	103	42.2
Totals	117	100.0	127	100.0	244	100.0

hemoglobin was 15.09 Gm. for those having pulse rates of 75 or less, 15.39 Gm. for those having pulse rates of 76 or more.) The Chi-square test applied to this four-fold table shows that a relationship of this extent could occur by chance less than once in 50 times ( $P = .02$ ). Arbitrarily, this is taken as a significant relationship. Comparison of Sahli hemoglobin with the hemoglobin by the Van Slyke method, with red blood cell count, with hematocrit shows much closer relationship than this, as would be expected. Hemoglobin level compared to each of the various factors that follow, however, never reached the probability of one in 50 ( $P = .02$ ), and rarely one in 20 ( $P = .05$ ).

Pulse rates during the physical examination determined in recumbent and standing positions also showed a tendency for the slower rates to accompany lower hemoglobin, faster rates higher hemoglobin, but not to the extent of sitting pulse rate and not significant by the described criterion. Such tendencies, however, help to confirm the general observation that hemoglobin is often higher in individuals with faster pulse rates. Since the pulse rate is a somewhat sensitive indicator of emotional state, it is likely that the hemoglobin in these men has also reflected to some extent emotional disturbance or excitement toward the examination and the drawing of blood, and although excitement was not a very marked response in these men, it was quite obvious in some cases. Increase of the red blood cells and

hemoglobin in excitement in man and animals has been described, the so-called "emotional polycythemia"<sup>13</sup> A group of the present subjects who have been judged by the psychiatrist to have greater than average instability of the autonomic

TABLE 3

Pulse rate	No subjects
Basal, compared to Sahli hemoglobin	178
Sitting before treadmill run,* compared to Sahli hemoglobin	125
Standing before treadmill run,* compared to Sahli hemoglobin	126
At start of treadmill run,* compared to Sahli hemoglobin	165
Maximum during treadmill run,* compared to Sahli hemoglobin	172
One minute after treadmill run,* compared to Sahli hemoglobin	171
Two minutes after treadmill run,* compared to Sahli hemoglobin	171
Four minutes after treadmill run,* compared to Sahli hemoglobin	172
Sitting compared to O <sub>2</sub> Capacity	140
Recumbent compared to O <sub>2</sub> Capacity	80
Basal compared to O <sub>2</sub> Capacity	117
Sitting compared to red blood cells	245
Recumbent compared to red blood cells	253
Standing compared to red blood cells	188
Sitting compared to hematocrit	244
Recumbent compared to hematocrit	251

\* Treadmill run refers to a standardized work experiment, carried out in the Harvard Fatigue Laboratory, a description of which may be found in reference 14

TABLE 4

	No subjects
Systolic B P, recumbent, compared to Sahli hemoglobin	257
Systolic B P, recumbent, compared to O <sub>2</sub> Capacity	84
Systolic B P, sitting, compared to Sahli hemoglobin	245
Systolic B P, standing, compared to Sahli hemoglobin	187
Diastolic B P, recumbent, compared to Sahli hemoglobin	257
Diastolic B P, sitting, compared to Sahli hemoglobin	243
Diastolic B P, standing, compared to Sahli hemoglobin	197
Pulse Pressure, recumbent, compared to Sahli hemoglobin	256
Systolic B P one minute after treadmill run compared to Sahli hemoglobin	66
Systolic B P sitting compared to red blood cells	246

nervous system functions showed a tendency to have higher hemoglobins (as well as higher pulse rates) than those not so judged. On the other hand, no significant relationship was seen between hemoglobin level and a medical estimate of general reaction to stress or of immediate reaction to venipuncture.

The additional comparisons shown in table 3 were made in the attempt to throw further light on the question of the relationships between the blood and pulse rate or emotional excitement.



TABLE 5

	No subjects
<i>Socio-economic factors</i>	
Location of birth compared to Sahli hemoglobin	250
Order of birth* compared to Sahli hemoglobin	256
Family income compared to Sahli hemoglobin	255
<i>Dietary† and gastro-intestinal factors</i>	
Daily calories compared to Sahli hemoglobin	253
Daily protein in food compared to Sahli hemoglobin	253
Daily meat in food compared to Sahli hemoglobin	253
Daily fat in food compared to Sahli hemoglobin	253
Per cent fat in food compared to Sahli hemoglobin	253
Daily carbohydrate in food compared to Sahli hemoglobin	253
Daily iron in food compared to Sahli hemoglobin	253
Daily calcium in food compared to Sahli hemoglobin	253
Daily phosphorus in food compared to Sahli hemoglobin	253
Daily glasses of milk compared to Sahli hemoglobin	253
Daily Vitamin C in food compared to Sahli hemoglobin	253
Daily Vitamin D in food compared to Sahli hemoglobin	253
Estimate of appetite compared to Sahli hemoglobin	235
Daily candy consumption compared to Sahli hemoglobin	245
Speed of eating compared to Sahli hemoglobin	182
Effect of stress on G I function compared to Sahli hemoglobin	250
Hemorrhoidal tabs compared to Sahli hemoglobin	254
Frequency of bowel movements compared to Sahli hemoglobin	252
Regularity of bowel movements compared to Sahli hemoglobin	242
<i>Physiologic and other factors</i>	
Duration of treadmill run compared to Sahli hemoglobin	176
Lactic acid after run compared to Sahli hemoglobin	84
Recovery Index (treadmill) compared to Sahli hemoglobin	171
Basal metabolism compared to Sahli hemoglobin	171
Basal metabolism compared to O <sub>2</sub> Capacity	68
Mouth temperature compared to Sahli hemoglobin	250
Respiratory rate (medical exam ) compared to Sahli hemoglobin	258
Respiratory rate (basal) compared to Sahli hemoglobin	202
Tidal air compared to Sahli hemoglobin	206
Blood groups compared to Sahli hemoglobin	200
Blood groups compared to O <sub>2</sub> Capacity	114
Frequency of past illnesses compared to Sahli hemoglobin	254
Number of dental fillings compared to Sahli hemoglobin	231
Dental occlusion compared to Sahli hemoglobin	243
Dental eruptions (maturation) compared to Sahli hemoglobin	245
Integration of personality compared to Sahli hemoglobin	186
Bland versus vital personalities compared to Sahli hemoglobin	97
Mood swings trait compared to Sahli hemoglobin	259
Soundness of personality compared to Sahli hemoglobin	245
Date of blood examination compared to Sahli hemoglobin	257

TABLE 5—*Continued*

	No. subjects
<i>Body-build factors</i>	
Height compared to Sahli hemoglobin	253
Weight compared to Sahli hemoglobin	253
Height/ $\sqrt{\text{weight}}$ compared to Sahli hemoglobin	252
Surface Area compared to Sahli hemoglobin	253
Head circumference compared to Sahli hemoglobin	252
Chest circumference compared to Sahli hemoglobin	252
Head circ /Chest circ compared to Sahli hemoglobin	252
Umbilical circ /Chest circ compared to Sahli hemoglobin	204
‡Pyknic component compared to Sahli hemoglobin	253
Somatic component compared to Sahli hemoglobin	253
Leptic component compared to Sahli hemoglobin	253
Predominance of somatotype compared to Sahli hemoglobin	179
‡Masculine component compared to Sahli hemoglobin	253
‡Masculine component compared to O <sub>2</sub> Capacity	152
‡Masculine component compared to hematocrit	252
‡Masculine component compared to red blood cells	254
‡Posture compared to Sahli hemoglobin	213

\* Order of birth was divided into (1) only child or first-born, (2) second-born, (3) third-born or later in rank of birth

† Dietary information was obtained from diet history of servings consumed and weighed average servings in the dormitory (See reference 1, page 128)

‡ There was a tendency, not significant, for the more pyknic (i.e. greater fatness and roundness and the less masculine body-builds to have somewhat higher blood levels

None of these comparisons showed a significant relationship according to the Chi-square test. The *tendency* was, however, for faster pulse rates to be associated with higher blood levels, in all instances but the two when recumbent and standing pulse rates were compared to red blood cell count. The consistency of these trends confirms the impression that higher blood levels tend to be found among individuals with faster pulse rates. It is possible, of course, that under the conditions of these observations, a type of emotional response in some individuals may result in lower blood levels with elevated pulse rates. It is worth noting that the trends became less significant as the effect of exercise on the pulse came into evidence. No relationship between work performance and hemoglobin could be established.

Comparisons with blood pressure were made as shown in table 4. Although no significance could be attached to the findings, the trends were consistent enough to suggest the predominance of lower hemoglobin levels in individuals having higher systolic blood pressures, and higher hemoglobin levels in those with higher diastolic pressures.

The comparisons given in table 5 were made in order to explore the possible relationships between socio-economic, dietary, physiologic, medical and body-build factors with blood levels. None of the comparisons showed significant relationships and no conclusions can be drawn other than that the hemoglobin level seems to be independent of these factors in healthy college men.

## COMMENT

Such negative findings in a relatively homogeneous group of young men should not be unexpected. They do not necessarily mean that relationships of hemoglobin with various environmental factors such as nutrition and disease incidence would not be present in larger population studies. Certainly, there have been shown to be distinct ranges of hemoglobin at different ages, in the two sexes, and in certain geographic areas where there are different nutritional influences. The relatively large range of hemoglobins in healthy young men remains unexplained by the present study. Emotional factors may be a partial explanation but future study will have to show the relative place of this and other factors.

## SUMMARY

1. The distribution of hemoglobins (obtained by the Sahli method on venous blood and by the  $O_2$  capacity method of Van Slyke) and the red blood cell counts of 153 to 259 college men, selected for participation in the Grant Study, are reported.

2. There is a statistically significant relationship between the hemoglobin and the pulse rate (those subjects having higher pulse rates tending to have higher hemoglobins). This relationship is apparently associated with emotional factors.

3. No significant relationship could be established between hemoglobin level and a variety of other factors, including socio-economic, dietary, physiologic, medical, body-build and others.

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# STUDIES OF THE STAPHYLOCOAGULASE REACTION NATURE AND PROPERTIES OF A PLASMA ACTIVATOR AND INHIBITOR

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SINCE Loeb's<sup>1</sup> observation in 1903 that certain strains of staphylococci possessed the property of clotting oxalated plasma, considerable study has been devoted to the mechanism of the staphylocoagulase reaction and to its possible role in staphylococcal infections. The ability to clot oxalated plasma has been found to be a property generally associated with pathogenic strains of staphylococci, however, the relationship between clotting activity and pathogenicity has not been clearly understood. Studies of the mechanism of the clotting action of staphylocoagulase have yielded several conflicting hypotheses.

Gengou<sup>2</sup> and Vanbreuseghem<sup>3</sup> noted that the clots produced from plasma by staphylococci underwent dissolution. Purified fibrinogen preparations were not clotted but underwent lysis without clot formation. In view of these observations, both authors agreed that the coagulant activity of staphylococci could be attributed to a fibrinolysin, whose action resulted in clotting only in the presence of a suitable plasma inhibitor. Gratia<sup>4</sup> proposed the term coagulase for the clotting agent elaborated by staphylococci and reported that the participation of proserozyne (prothrombin) or cytozyme (thromboplastin) was not necessary for the action of coagulase, plasma freed of these substances by adsorption methods remained clottable. He also noted that purified fibrinogen could not be clotted by staphylococci, but clotting occurred readily when a small amount of a plasma preparation was added, which had been freed of its content of fibrinogen, proserozyne, and cytozyme. Gratia concluded that the clotting activity of staphylococci was due to the elaboration of coagulase, but for the elaboration of this agent a nutrient factor was required which was not present in purified fibrinogen preparations, but was present in plasma. However, in opposition to these reports, Cruickshank<sup>5</sup> and Walston<sup>6</sup> both reported that coagulase acted on fibrinogen directly, and was therefore similar in its action to thrombin. In the most recent studies, Smith and Hale<sup>7</sup> confirmed the earlier reports and showed that fibrinogen was not clotted by cell-free coagulase unless there were added small amounts of human or rabbit plasma, serum, or testicular extract. The accessory clotting factor present in the plasma and testicular extract was not identical with either prothrombin or thrombokinase. The authors proposed that the function of this factor was to convert staphylocoagulase into an active thrombin-like substance and they therefore termed it activator. It was also suggested that the relative insusceptibility of the plasmas of certain animal species to the clotting action of coagulase could be attributed to a deficiency of activator in these plasmas.

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Dr. Robert Pennell and Dr. W. F. Verwey of the Medical Research Division, Sharp and Dohme, prepared and supplied several preparations used in these studies.

The occurrence in the serum of a factor which participated in the coagulase reaction was also observed in this laboratory, confirming the report of Smith and Hale.<sup>7</sup> This factor has been extracted from human plasma, and its nature and properties examined in an attempt to elucidate further its role in the clotting mechanism. In addition, it has also been observed that an inhibitor for coagulase could be obtained from normal human plasma and from the plasma of certain species of animals. This inhibitor appeared to play a significant role in determining the susceptibility of these plasmas to the action of coagulase. In the present report, the properties and action of the activator and inhibitor for coagulase are described.

### MATERIALS AND METHODS

1 *Staphylocoagulase* The source of coagulase throughout this study were viable cultures of coagulase positive strains of staphylococci which had been grown in tryptose-phosphate broth for 48 hours.

2 *Fibrinogen* The fibrinogen preparation\* employed was a dried product which had been precipitated repeatedly from pooled normal human plasma by the method of alcoholic fractionation.<sup>8</sup> A 2 per cent solution of the dried powder was used.

3 *Thrombin* The preparation was a 1:10 dilution of a commercial product derived from rabbit blood.<sup>†</sup>

4 *Plasma defibrinated by heating* Oxalated or citrated plasma was heated to 56°C, kept at this temperature for three minutes, and then immediately cooled in running water. The flocculated fibrinogen was removed by centrifuging. The defibrinated plasma thus obtained possessed no thrombic activity for fibrinogen.

5 *Plasma coagulase test* Two tenths ml. of a forty-eight hour culture of a given strain of staphylococcus was added to 0.5 ml. of citrated or oxalated plasma, and the tube placed in a water bath at temperature 37°C. Periodic observations were made to note the time and degree of clotting. A test was considered positive only if a cohesive clot was formed within twenty-four hours, while a test was considered negative if no clot was formed after incubation for twenty-four hours.

### EXPERIMENTAL

#### I PARTICIPATION OF A PLASMA FACTOR IN THE STAPHYLOCOAGULASE REACTION

Fresh human plasma has been generally employed as a substrate for use in the routine staphylocoagulase test. Because of the availability, however, of such products as frozen plasma, dried plasma, and dried fibrinogen, it was of interest to determine whether these materials could be substituted for fresh plasma in the test. Accordingly, a series of pathogenic strains of staphylococci were tested with these substrates as well as with fresh human plasma. Using fifteen different strains as a source of coagulase, it was found that the substitute substrates were much less susceptible to coagulation than the fresh plasma. As shown in table 1, of the fifteen strains which were coagulase-positive when tested with fresh human plasma, only seven clotted plasma which had been rethawed from the frozen state, three clotted reconstituted dried plasma, and none clotted solutions of lyophilized fibrinogen even after incubating for a period of twenty-four hours. Moreover, in those instances in which clotting of the frozen or dried samples of plasma occurred, the clotting time was prolonged, ranging from twelve to twenty-four hours as contrasted with one to two hours for samples of fresh plasma. The relative refractoriness of these three substrates to clotting by coagulase could not be attributed to a deficiency of clottable fibrinogen since all three substrates were readily clotted by such agents as thrombin, snake venom, and papain within two to eight minutes.

\* The fibrinogen preparation was kindly supplied by Sharp and Dohme, Inc.

† Hemostatic Globulin, Lederle Laboratories, Inc.

A possible explanation for the apparent resistance of these samples of fibrinogen and plasma to clotting was that they were deficient in some clotting factor present in fresh plasma. Prothrombin was excluded as this factor since the Quick<sup>9</sup> prothrombin levels of both the frozen and dried plasma were practically the same as that of fresh plasma, 1 e, 12.8, 12.3, and 12.5 seconds respectively. Tests for an accessory clotting factor were therefore carried out with fresh human serum. As a test substrate, 0.2 ml of fresh human serum was mixed with 0.5 ml of fibrinogen, and susceptibility to clotting determined by addition of 0.2 ml of a staphylococcus culture. It was at once apparent that the addition of serum rendered the fibrinogen readily clottable by coagulase. Further, the clotting which resulted was not due to the presence of thrombin in the serum, since interference by thrombin could be excluded by suitable dilution of the serum or by destroying its thrombin activity.

TABLE 1—*Comparative susceptibility of fresh plasma, frozen plasma, dried plasma, and dried fibrinogen to clotting by 15 strains of pathogenic staphylococci*

No	Strain of Staphylococcus	Coagulase Test			
		Fresh Plasma	Frozen* Plasma	Dried Plasma	Dried Fibrinogen
1	Wro	+	+	+	—
2	May	+	—	—	—
3	St A	+	+	—	—
4	Lin	+	—	—	—
5	Ver	+	+	—	—
6	Sha	+	—	—	—
7	Sch	+	+	+	—
8	Lon	+	+	+	—
9	Slo	+	—	—	—
10	Ter	+	—	—	—
11	Cos	+	—	—	—
12	Ede	+	—	—	—
13	Pew	+	+	—	—
14	Rut	+	—	—	—
15	Nel	+	+	—	—

\* This plasma was stored in the frozen state for two years before thawing

by heating at 60 C for thirty minutes. These procedures still permitted the activity of the accessory coagulase factor to be readily demonstrated.

The most satisfactory source of this accessory factor was plasma from which the fibrinogen was removed either by heating to 56 C for three minutes, or by precipitation at one-fourth saturation with ammonium sulfate. These preparations had the advantage that they possessed no thrombin activity. As shown in table 2, when 0.2 ml of such a defibrinated sample of plasma was added to fibrinogen, clotting occurred almost as readily as with the fresh plasma. Fibrinogen to which no defibrinated plasma had been added was not clotted by any of these strains. This failure to clot could not be attributed simply to the destruction of the fibrinogen by the fibrinolytic action of the staphylococci, as had been suggested by Gengou.<sup>2</sup> Indeed, of the five strains included in table 2, only two were found to

have destroyed the fibrinogen at the end of twenty-four hours as was indicated by the fact that the addition of thrombin did not result in a clot. The other three strains apparently exerted no appreciable fibrinolytic effect in the twenty-four hour period, since fibrin clots were obtained following the addition of thrombin. Similar results have been obtained with a larger series of tests and it was therefore concluded that the failure of preparations of fibrinogen to be clotted by coagulase was not due to the action of a fibrinolysin but rather to the deficiency of an accessory clotting factor.

This clotting factor, as it has been previously pointed out, has been termed an activator of coagulase by Smith and Hale.<sup>7</sup> In the present state of knowledge,

TABLE 2.—*The effect of addition of defibrinated plasma\* on the clotting of fibrinogen by coagulase*

Strain of <i>Staph. aureus</i>	Substrate Mixture				Clotting time
	Plasma	Fibrinogen	Defibrinated Plasma	Saline	
	ml	ml	ml	ml	hr
Sch	0.5			0.3	1
Sch		0.5		0.3	>24 (c)†
Sch		0.5	0.3		2
Rut	0.5			0.3	1
Rut		0.5		0.3	>24 (c)
Rut		0.5	0.3		2
Bur	0.5			0.3	1
Bur		0.5		0.3	>24 (nc)
Bur		0.5	0.3		2
Ter	0.5			0.3	1
Ter		0.5		0.3	>24 (c)
Ter		0.5	0.3		2
Lou	0.5			0.3	1
Lou		0.5		0.3	>24 (nc)
Lou		0.5	0.3		2

\* The defibrinated plasma was prepared by heating normal human plasma at 56 C. for 3 minutes.

† (c) represents clot formation on addition of thrombin and (nc) represents no clot formed on addition of thrombin.

such a function should be regarded as highly hypothetical, however, for purposes of uniformity the term activator has been retained in the present report.

## 2. TESTS OF PLASMA FRACTIONS FOR ACTIVATOR CONTENT

In order to determine which of the plasma protein fractions contained the activator, human citrated plasma was fractionated into five components at increasing saturation with ammonium sulfate, and each fraction then tested for its content of activator. Precipitates were obtained at 25, 40, 60, 80, and 100 per cent saturation with ammonium sulfate. Each precipitate was washed once in the appropriate concentration of ammonium sulfate, and redissolved in distilled water to a volume one-half that of the original plasma. The solutions were then dialyzed against physiological saline solution in the cold for eighteen to twenty-four hours.

The method of titration for activator was as follows. Serial dilutions with physiological saline solution were made with each fraction in 0.5 ml. volumes. To each dilution was added 0.5 ml. of a forty

eight hour broth culture of a standard strain of staphylococcus (Bur ), and incubation carried out for one hour in a water bath at 37 C To each tube was added 0.5 ml of fibrinogen solution, the tubes shaken, and incubation carried out for four hours The titer of activator in the sample was taken as the highest dilution which yielded a solid cohesive matrix clot

As shown in table 3, the activator was concentrated essentially in the fraction obtained between 80 and 100 per cent saturation, while the fraction between 60 and 80 per cent saturation possessed smaller amounts of activity Activator thus appeared to be associated with the crude albumin fraction of the plasma

A more precise characterization of the nature of activator was made possible by testing human plasma fractions prepared by alcoholic fractionation \* It has been shown by Cohn and collaborators<sup>8</sup> that the plasma proteins may be separated into five fractions on the basis of their differential solubility in ethanol-water mixtures at low temperatures and at varying pH and ionic strength These five fractions, I, II + III, IV-1, IV-4, and V, as well as a sample of crystalline serum albumin, were

TABLE 3—Activator titer of human plasma fractions prepared by ammonium sulfate precipitation

No	Plasma Fractions	Titer* of Activator
1	25% sat $(\text{NH}_4)_2\text{SO}_4$	0
2	25-40% $(\text{NH}_4)_2\text{SO}_4$	0
3	40-60% $(\text{NH}_4)_2\text{SO}_4$	0
4	60-80% $(\text{NH}_4)_2\text{SO}_4$	4
5	80-100% $(\text{NH}_4)_2\text{SO}_4$	16

\* Highest dilution of the test substance which when mixed with fibrinogen permitted clotting by coagulase in four hours

titrated for their relative content of activator The solutions in each case were made up to contain 200 mg of the dried fraction in 10.0 ml of 0.1 M phosphate buffered saline, pH 7.4

As shown in table 4, activator was found to a significant extent only in fractions IV-4 and V The greater amount of activity appeared in fraction IV-4, which is made up chiefly of alpha and beta globulins Fraction V, which is essentially albumin, contained a somewhat smaller amount of activator, while crystalline albumin was without activity

From the above findings, it appeared that activator is probably not an albumin although it is precipitated with this protein fraction by ammonium sulfate In plasma fractions obtained by alcoholic fractionation, activator appeared to be associated with the alpha and beta globulins

### 3 PREPARATION OF DRIED ACTIVATOR MATERIAL FROM CITRATED HUMAN PLASMA

The activator preparations used in the subsequent studies were prepared from the fraction of plasma precipitated between 60 and 100 per cent saturation with

\* These fractions were supplied by Dr E J Cohn and his associates The materials were made possible through contracts between Harvard University and the Office of Scientific Research and Development and the plasma was obtained through the American Red Cross and more recently through the Massachusetts Civilian Blood Donor Program



ammonium sulfate To 1 volume of fresh citrated human plasma was added slowly with continuous stirring 1.5 volumes of saturated ammonium sulfate, and the mixture allowed to stand for four hours at temperature 4 C This precipitate obtained at 60 per cent saturation was removed by filtration in the cold and discarded To the supernate, solid ammonium sulfate was added to saturation, and the mixture was usually allowed to stand overnight in the refrigerator before filtering The fraction thus obtained between 60 and 100 per cent saturation was dissolved in a small amount of distilled water and dialyzed against physiological saline solution for eighteen hours in the cold The resulting solution was then dried from the frozen state by the lyophile process and the resulting light yellow powder used

TABLE 4 — *Activator titer of human plasma fractions obtained by alcoholic fractionation*

No	Alcoholic Fraction	Titer* of Activator
1	I	0
2	II + III	0
3	IV-1	0
4	IV-4	8
5	V	2
6	Crystalline albumin	0

\* Highest dilution which when mixed with fibrinogen solution permitted clotting by coagulase in four hours

TABLE 5 — *Thermolability of Activator*

No	Temperatures at which preparations were exposed for 30 min	Titer of Activator	
		Defibrinated Plasma	Activator Preparation (Am. Sulfate Fraction)
1	Control (not heated)	8	8
2	55 C	8	8
3	65 C	8	4
4	75 C	8	2
5	100 C	4	2
6	Boiling	0	0

without further purification It was found to be completely stable for at least a month when stored in the cold Solutions of activator employed in the present study were usually made up in a concentration of 200 mg of dried preparation per 10 ml of distilled water

#### 4 PROPERTIES OF ACTIVATOR

A *Thermolability* Smith and Hale<sup>7</sup> found the activator present in rabbit testicular extracts to be completely inactivated after heating at 56 C for thirty minutes Such thermolability, however, has not been observed with activator derived from human plasma Heat sensitivity tests, using both defibrinated plasma and am-

monium sulfate fractions as a source of this material, have indicated no apparent inactivation following exposure to 56 C for thirty minutes. Indeed, as shown in table 5, complete inactivation of defibrinated plasma did not occur even after heating at 100 C for thirty minutes, although there was some loss of activity. Strong boiling for thirty minutes caused complete destruction.

*B Stability at various pH* Solutions of activator were markedly stable within a wide range of acid and alkaline pH. The effect of pH variation was determined by mixing given amounts of activator with a series of buffers from pH 2.0 to 10.0 and then permitting these mixtures to stand for four hours at room temperature before neutralizing. The mixtures thus treated showed no measurable loss of activator titer.

*C Inactivation by pepsin and nitrous acid* Since activator was stable at acid pH, it was possible to determine whether it could be destroyed by the proteolytic action of pepsin. Accordingly, 1.0 ml of activator solution was adjusted to pH 3.0, and incubated with 1.0 ml of a 0.05 per cent solution of pepsin (Merck) for

TABLE 6—*The inactivation of activator by treatment with (1) pepsin and (2) nitrous acid*

Expt No	Reaction Mixture	Activator Titer
1	(a) Activator + pepsin	0
	(b) Activator + saline control	8
2	(a) Activator + nitrous acid	0
	(b) Activator + saline control	4

thirty minutes at 37 C. At the end of this period, pepsin action was stopped by neutralizing the mixture with  $\frac{1}{2}$  M phosphate buffer, pH 7.5. The resulting mixture was then titrated for its activator content. As shown in table 6, activity of activator was completely lost by this treatment, while control samples, in which saline solution was substituted for pepsin and which were subjected to the same temperature and pH, remained fully active.

Treatment of activator solutions with nitrous acid also resulted in rapid inactivation. On addition of dilute sodium nitrite solution to activator preparation at pH 3.0, complete inactivation was observed within fifteen minutes (table 6). Since the action of nitrous acid within this period is primarily directed against alpha-amino groups, it would appear that the action of activator is dependent upon the integrity of alpha-amino groups in the molecule.

The data obtained would indicate that activator is a relatively stable protein constituent of the plasma. It is precipitated between 60 and 100 per cent saturation with sulfate, but in alcoholic fractions it is associated more closely with the alpha and beta globulins. It is nondialyzable, is not readily destroyed by heating below 100 C, and is entirely stable between pH 2.0 to 10.0. Its activity is completely destroyed by nitrous acid or by the proteolytic action of pepsin.

### 5 PRESENCE IN NORMAL HUMAN PLASMA OF A SUBSTANCE INHIBITING THE COAGULASE REACTION

It was noted that when some samples of human defibrinated plasma were employed as a source of activator, clot formation occurred much more rapidly when the samples were diluted. This same accelerating effect was observed also on diluting some activator preparations. One given activator preparation, for example, when mixed with fibrinogen and coagulase, permitted clotting to occur in eight hours, but when this same preparation was diluted 16-fold, clotting occurred in two hours. This observation suggested the presence in these activator preparations of an inhibitor which interfered with clot formation, and that the action of the inhibitor could be suppressed by suitable dilution.\* It was noted, however, that the inhibitory effect did not occur with all preparations of activator.

On fractionation of human oxalated plasma by means of ammonium sulfate, the inhibitor was found to be present in the globulin fraction obtained at half-saturation. The inhibitory action of such a globulin preparation is demonstrated in the following experiment. One-tenth ml of a 4 times concentrated human globulin preparation was added to a mixture containing 0.5 ml of activator solution, 0.5 ml of coagulase and 0.5 ml of fibrinogen, and the clotting time noted. The result

TABLE 7 — *Inhibitory effect of a plasma globulin factor on the coagulase reaction*

Globulin	Activator	Coagulase	Saline	Clotting Time
ml	ml	ml	ml	hrs
0.1	0.5	0.5	0	>24
0	0.5	0.5	0.1	2

was compared with a control test, in which 0.1 ml of saline solution was substituted for the 0.1 ml of test preparation. As shown in table 7, the mixture containing the plasma globulin preparation was not clotted even after twenty-four hours, while a clot appeared in the control tube in two hours. The coagulase reaction was thus entirely inhibited by the globulin preparation.

In order to compare quantitatively the content of inhibitor in different plasma fractions, a method was devised for determining inhibitory titer. The inhibitory titer of a given sample was measured as follows. Serial dilutions of the test sample were made in saline solution in 0.5 ml volumes. To each tube, 0.5 ml of activator solution and 0.3 ml of coagulase was added, and these mixtures were then incubated for sixty minutes. Finally, 0.5 ml of fibrinogen was added, and the tubes incubated for four hours. The highest dilution of the test sample preventing clotting completely at the end of this four hour period was taken as the inhibitory titer.

Table 8 demonstrates that practically all of the inhibitor present in human plasma is associated with the fraction obtained at 25 to 40 per cent saturation with ammonium sulfate. When such globulin preparations were dialyzed against dis-

\* The greater susceptibility to clotting of plasma which is diluted also led Lominski and Roberts to the recognition of an inhibitor in the plasma which they have independently described.<sup>10</sup>

tilled water, the inhibitor was found to be associated with the insoluble euglobulin fraction, while the pseudoglobulin fraction contained little or no inhibitory action

It appeared possible that the presence of this globulin inhibitor in high concentration might be in part responsible for the resistance to the action of coagulase observed in some human plasmas,<sup>6</sup> and in the plasmas of certain species, such as the guinea pig,<sup>3</sup> mouse, and fowl.<sup>7</sup> Smith and Hale<sup>7</sup> stated that the resistance of these plasmas might be explained by their deficiency of activator. The participation of an inhibitor was not recognized.

In order to determine the relationship of the inhibitor to coagulase susceptibility, tests were carried out for the presence of inhibitor in both susceptible and resistant plasmas. Human and rabbit blood was used as a source of susceptible plasma, while the resistant plasma was obtained from the guinea pig. Previous studies have indicated that, of these three species, rabbit plasma is the most susceptible.<sup>11</sup> Guinea pig plasma is unusual in that it is not clotted at 37 C, but is clotted slowly at 25 C.<sup>3, 7</sup>

TABLE 8—*Titer of inhibitor in human plasma fractions*

	Plasma Fraction Tested	Dilution of Fraction					
		1	2	4	8	16	32
1	25-60% sat (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	o	o	o	o	+	+
2	25-40% sat (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	o	o	o	o	+	+
3	40-60% sat (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	+	+	+	+	+	+
4	60-100% sat (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	+	+	+	+	+	+
5	Pseudoglobulin	+	+	+	+	+	+
6	Euglobulin	o	o	o	o	+	+

+ = clot formed, o = no clot formed

The susceptibility of each plasma was tested by the routine coagulase test both at 37 C and at 20 C. It was found that at 37 C the clotting of human and rabbit plasmas occurred in thirty minutes, while clotting of guinea pig plasma occurred in sixty minutes. At 20 C, the human and rabbit plasmas were clotted in two hours, while guinea pig plasma was clotted in eight hours.

Thus, in this study, the effect of temperature was precisely the reverse of that previously reported. Guinea pig plasma was definitely clotted by the strain of staphylococcus employed, and the clotting occurred more rapidly at 37 C. However, this plasma was still less susceptible than the plasmas of the other two species.

The inhibitor content of each plasma was determined by testing the extracted globulin fraction, since it was desired to avoid any possible interference resulting from the presence of activator in the whole plasma. Each sample was first defibrinated at 56 C, and the globulin precipitate then separated from the resulting sample by half-saturation with ammonium sulfate. The precipitate was removed by filtration and was dissolved in distilled water, and the volume brought up to that of the plasma originally used. Each preparation was then titered for its inhibitor content.

As shown in table 9, the rabbit preparation contained no inhibitor by the method employed, while both the human and guinea pig globulin preparations possessed a titer of 8. The absence of inhibitor in the rabbit was interesting in view of the marked susceptibility of the plasma of this species, however, an inverse relationship between inhibitor level and susceptibility did not seem to be general as was indicated by the similar levels of inhibitor in human and guinea pig plasmas.

Since susceptibility must depend also on the presence of activator in the plasmas, the plasma of each of these three species was next analyzed for their content of activator. Titrations were performed both on defibrinated samples and on the albumin fractions separated between 60 and 100 per cent saturation. Each albumin precipitate was dissolved in distilled water and brought up to the original plasma volume, so that the concentration of activator was presumably the same as that in the plasma.

As shown in table 9, the activator titer of the guinea pig plasma was significantly lower than either the human or rabbit plasma. Thus, the relative titers of activator and inhibitor in the plasmas of these species appeared to explain their degree of susceptibility to the action of coagulase. Rabbit plasma which was most susceptible

TABLE 9—*The levels of inhibitor and activator in human, rabbit, and guinea pig plasmas*

Plasma of Species Tested	Inhibitor Titer of Globulin Fraction	Activator Titer of Defibrinated Plasma	Activator Titer of Albumin Fraction
Human	8	8	8
Rabbit	0	16	8
Guinea Pig	8	4	2

contained a high level of activator and no inhibitor, while guinea pig plasma which was less susceptible than that of the rabbit contained less activator and a more elevated level of inhibitor. The susceptibility of the human plasma, which also contained an elevated level of inhibitor may perhaps be explained by the high level of activator which it possessed.

The action of the inhibitor did not seem to be specifically exerted on activator. This conclusion followed from the repeated observations that plasma samples could be readily separated into inhibitor-containing and activator-containing fractions, the activities of which were entirely independent of each other. Further, as shown in table 9, the separation of activator from defibrinated plasma did not result in an increase in the titer of activator, as might be expected, if activator were freed from an inhibitor. The evidence, therefore, suggests that the inhibitor does not combine with activator, but acts on some other factor in the coagulase reaction, presumably coagulase.

#### DISCUSSION

The present report has confirmed previous observations in that the coagulase reaction involves the participation of a plasma factor which has heretofore not been described. Dried plasma and plasma stored in the frozen state for long periods

are not readily clotted by coagulase since they contain reduced amounts of this reactive factor. The present studies have demonstrated that this plasma factor is a protein which is precipitated with the crude albumin fraction by means of ammonium sulfate, but it appears to be more closely associated with the alpha and beta globulins in alcoholic fractions.

Smith and Hale<sup>7</sup> have proposed that the clotting factor in plasma and testicular extracts is an activator of coagulase. The reaction is held analogous to the activation of thrombin by thrombokinase plus calcium ions. In this regard, the present observation that activator is a protein of considerable stability is not inconsistent with the possibility that it possesses an activating function analogous in some respects to thrombokinase.<sup>9</sup> However, it is equally probable that activator is the substrate, and that coagulase is the activator. Indeed, it is also entirely possible that no activation occurs but that an interaction of a different nature between coagulase, plasma factor, and fibrinogen is involved which is responsible for the precipitation of fibrin. A study of the chemical kinetics of the reaction is highly desirable, but must await the purification and concentration of these three reactants.

In the course of these studies, it was noted that a globulin could be separated from human plasma which inhibited the staphylocoagulase reaction. The inhibitor was also found in the normal plasma of the guinea pig but was absent in rabbit plasma. Previous studies<sup>6, 12, 13</sup> have reported that some human plasmas appear to be resistant to the action of coagulase, and have suggested that such resistance may be attributed to the presence of a specific antibody to coagulase. The recent report of Lominski and Roberts<sup>10</sup> has also favored the antibody hypothesis. These authors observed that the inhibitor was a globulin, that it was heat-stable, and that it combined specifically with coagulase. They concluded that these findings could only be consistent with an antibody hypothesis. In opposition to this hypothesis, however, are the reports<sup>6, 7</sup> that attempts to immunize animals with coagulase or to demonstrate in man the development of resistance to coagulase following staphylococcal infection<sup>5, 13</sup> have met with failure. It is, of course, possible that the failure to demonstrate the development of resistance in these cases was due to the inadequate methods employed for measuring the inhibitor as Lominski and Roberts<sup>10</sup> suggest. If, then, it be assumed that the antibody hypothesis is correct, the present observations that the inhibitor is present in the plasma of normal guinea pigs, and entirely absent in the normal plasma of the rabbit may be explained only by the assumption that the guinea pig acquires the antibody as a result of a natural or silent immunization, while the rabbit is immunologically insensitive. More exact immunologic studies are required to answer this problem.

The presence of the inhibitor in the plasma of the guinea pig, which has been found resistant to coagulase, and its absence in rabbit plasma which is highly susceptible, suggests that this factor may play an important role in determining species susceptibility. Smith and Hale<sup>7</sup> have reported that the resistance observed with different species may be attributed to a deficiency of activator. However, it is probable that both activator and inhibitor are the variables which determine

susceptibility It should be noted, that since the inhibitor apparently combine with coagulase and not with activator, an excess amount of coagulase would produce clotting even in the presence of inhibitor, provided some activator were present The staphylocoagulase test is then dependent on the quantitative interrelationships of the three variables—coagulase, activator, and inhibitor The variation in susceptibility which is observed when plasmas of various species are tested with different strains of staphylococci may be a quantitative phenomenon which is dependent upon the relative amounts of coagulase, activator, and inhibitor participating in these clotting systems

### CONCLUSIONS

Dried human plasma and human plasma which has been stored in the frozen state for long periods are not readily clotted by coagulase Fibrinogen which is sufficiently purified is entirely insusceptible to clotting by coagulase However, all three substrates—dried human plasma, frozen plasma, and dried human fibrinogen—are readily clotted by coagulase on admixture of a plasma factor

This accessory substance, referred to as activator, is precipitated from normal human plasma at 60 to 100 per cent saturation with ammonium sulfate However, it is not present in purified serum albumin preparations, but does occur in alcoholic fractions which largely contain alpha and beta globulins Activator is a comparatively stable protein which resists heating at temperatures below 100 C, but is destroyed by pepsin or nitrous acid

A substance inhibiting the coagulase reaction was found present in many samples of normal human plasma It was associated with the globulin fraction It was also present in the normal plasma of the guinea pig but was deficient in rabbit plasma The inhibitor substance acts independently of activator and is therefore believed to inhibit the clotting reaction by its action on coagulase Either the presence of this inhibitor or a deficiency of activator may be responsible for the failure of coagulase to clot the plasmas of some individuals and of certain animal species.

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## SOME HEMATOLOGIC EFFECTS OF IRRADIATION\*

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DURING our study of the cellular changes in mammals exposed to external and internal sources of ionizing radiations, evidence was sought for correctness of the idea that small doses of radiations stimulate cellular activity and multiplication. Although this idea is held by a number of radiologists, little basis for it is found in the radiologic literature other than misunderstanding and misquotation of a few important papers. Some of our experiments offered unusual opportunities for a consideration of the question of stimulation. Thus, in those experiments in which there were progressive decreases in the amount of irradiation and in the reactions to focalized irradiation, one could expect to find evidence of stimulating effects if they actually occurred. But our findings in the organs and peripheral blood have been so consistently at variance with the idea of stimulation by small amounts of ionizing radiation that it seems desirable to summarize these observations.

Some of the confusion on this question undoubtedly originates from a loose use of the word stimulation. In the following discussion we shall discriminate between a primary stimulation which results directly in cellular hyperplasia, hypertrophy, or hyperactivity after irradiation, without a stage of obvious previous injury, and a secondary stimulation which might be considered as a reparative process resulting from the necrotizing action of radiation.

### MATERIAL AND METHODS

The radiations which were employed in these studies on animals and man fall essentially into two main categories: (1) Externally originating total body irradiation from x,  $\gamma$  rays, fast and slow neutrons. (2) Focal irradiation by externally originating B rays and radioactive isotopes administered enterally or parenterally.

1. *Total body irradiation* The organs of large numbers of rabbits, rats, and mice and those from a smaller number of guinea pigs were obtained for histologic study when the animals were sacrificed at intervals after varying dosages of x and  $\gamma$  rays, fast and slow neutrons. The general plan of these experiments was to start with the LD 50/30 days dose of each agent and to decrease it until no histologic or hematologic changes were observed. In most of the series the total amount of irradiation was given at one exposure, although there were several large and important series in which small amounts of radiation were given repeatedly.

We found it necessary to carry out these experiments for histologic purposes by killing the animals at planned intervals because cytologic examinations of animals

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which were found dead were worthless. The series was supplemented with the study of a number of moribund animals.

2. *Focal irradiation* This occurred in the spleens and testes of a few mice given large amounts of irradiation by B rays from an external source of  $P^{32}$ . However, the great mass of our experiments with focalized irradiation effects occurred after the intravenous or intraperitoneal injection of radioactive isotopes. In a few series these materials were given intramuscularly or by inhalation. Among the elements studied were the  $\alpha$  (Alpha) emitters Ra and Pu, the  $\beta$  emitters  $P^{32}$ ,  $Sr^{89}$ ,  $Y^{91}$ , and the  $\beta$  and  $\gamma$  (Gamma) emitters  $Zr^{40}$ ,  $Ba^{140}$ ,  $La^{140}$ ,  $Na^{24}$ , and the degradation products of Ra. These isotopes were given in dosages starting with the LD 50/30 days and in diminishing fractions thereof.

The tissues were routinely fixed in Zenker-formol, imbedded in nitrocellulose and stained with hematoxylin-eosin-azure II, except for those portions of the tissues which were fixed in alcohol so that auto-radiographs could be prepared.

Whenever possible, studies of the peripheral blood were conducted on the same series of animals in which sacrifices were being made for cytologic study. Where this was not possible, groups of animals were studied in parallel series, in still other experiments, blood counts were made on the animal at the time of sacrifice in an attempt to correlate the peripheral hematologic picture with effects on the blood forming organs. Sampling of peripheral blood was done at intervals varying with the chronicity of the experiment. In all cases, the techniques employed were standard hematologic procedures.

From the standpoint of the hematologic studies, the experiments of most significance were (1) those in which an acute single dose of penetrating total body radiation was given (x-rays or fast neutrons) and (2) the experiments in which rats, guinea pigs, mice, and rabbits were exposed chronically to  $\gamma$  rays or x-rays. In one of the chronic gamma ray exposure experiments, the radiation was given daily over an eight hour period, and in another set of experiments, the  $\gamma$  radiation was given twenty-four hours per day. In the chronic x-ray experiments, the daily exposure was given within a few minutes. The daily exposure of these animals, whether exposed to gamma rays or x-rays, extended over long periods up to four years.

## OBSERVATIONS

### I. CHANGES AFTER A WIDE RANGE OF DOSES OF TOTAL BODY IRRADIATION FROM EXTERNAL SOURCES

One of the effects of total body external irradiation in doses not higher than the LD 50/30 days is to separate those organs which are sensitive to this amount of irradiation and those which are not. In the laboratory mammals which were studied with this dosage, the radiosensitive organs are the gonads, bone marrow, spleen, lymph nodes, thymus, parts of the gastrointestinal tract, skin and bone. Nerve, muscle, and most exocrine and endocrine glands are resistant.<sup>1</sup>

It was found that all of the external ionizing radiations in equivalent doses produced similar effects.<sup>1</sup> If the animals had been given the LD 50/30 days dose of x-rays, or  $\gamma$  rays, or fast or slow neutrons, the changes which resulted could not be

distinguished from one another. That is, in examining sections of the organs of these animals, we could not tell which ionizing radiation had been used. With this amount of irradiation the very first changes observed, usually within the first hour, were degenerative changes and death of cells in the blood forming organs, bases of the intestinal crypts and foveolae of the stomach, spermatogonia, and ovarian follicular epithelium or developing ova, and early mild inflammation (mainly edema) of skin.

Mitoses were usually gone by the second hour. There was no evidence of an increase in mitosis. In rabbit bone marrow after 100r of total body x-irradiation, mitoses began to be present again after three hours. They continued through the fourteen hour interval at a level slightly above that seen in controls. At the eight hour stage some of the mitoses were abnormal. During the next hours, the debris increased in amount and was gradually phagocytized. With an LD 50 dose after a few days the blood forming organs became markedly reduced in size and the bone marrow and lymph nodes became aplastic—often completely so.

In the course of one to two weeks the blood forming organs gradually became actively hematopoietic again. Only rarely did this process reach or exceed normal in the bone marrow. Several months usually elapsed before the lymph nodes, thymus, and spleen returned completely to normal.

With progressively smaller doses the degenerative effects were less marked. They became minimal at 50r of x-rays and were just detectible in our animals at 25r of x-irradiation or the equivalent fraction of the LD 50/30 days dose of neutrons. With doses lower than 25r, no changes were found, in animals so treated there was no evidence whatever for either an injurious or stimulating effect.

In the regeneration after external irradiation the only instances of overcompensation of tissue was in bone marrow of rabbits which survived the LD 50/30 days. In them the marrow seemed hyperplastic (both erythropoietic and myelopoietic within two months after exposure).

After the administration of single doses of either x-rays or fast neutrons to rabbits, mice, and rats, an increase in circulating heterophil-leukocytes occurred within the first twenty-four hours with doses up to and beyond LD 50/30 days. In fact, with doses of 500 and 800r in the rabbit Jacobson et al.<sup>2</sup> noted that two definite statistically significant peaks occurred at circa twelve and twenty-four hours. After doses of this magnitude, a leukopenia invariably followed the heterophil leukocytosis. With doses of 300r and below, only a modest elevation in the number of circulating heterophils occurred and this at about twelve hours. Control animals handled in a comparable manner (except for the actual exposure to irradiation) also had this latter modest peak increase after about twelve hours. Heterophils are the only circulating cells which are initially increased in number after acute total body radiation. Lymphocytes, monocytes, eosinophils, reticulocytes, and platelets are reduced.

As recovery occurs in the hematopoietic organs, however, a "compensatory increase" above normal control values was not uncommonly encountered, particularly for the heterophils and reticulocytes. No significant absolute lymphocyte increase on a compensatory basis was encountered in experiments in which doses

of 800r to 5r acute total body X radiation were administered. In fact, lymphocyte values in the peripheral blood of the rabbit are reduced over a period of ninety days after an acute exposure to 800r. This is in agreement with the slow return of the lymph nodes to normal after irradiation.

After acute doses of x-rays, or fast neutrons Jacobson et al.<sup>2, 3</sup> observed an "abortive rise" in lymphocytes, heterophils, and reticulocytes in rabbits between the fourth and twelfth day after irradiation. This rise may possibly represent an abnormal stimulation in the sense that certain precursors are sufficiently altered to produce a limited succession of abnormal progeny.

### *Chronic Radiation Experiments*

Chronic exposure of groups of rabbits, mice, and guinea pigs to  $\gamma$  rays in daily doses of 0.11, 1.1, 2.2, 4.4, 8.8  $\gamma$  for eight hours per day or twenty-four hours per day over periods extending beyond three years carried out by Lorenz et al.<sup>4</sup> has not shown evidence of a stimulating effect on the blood forming tissue which was reflected in the hematologic constituents of the peripheral blood. Similar experiments were conducted with chronic daily exposure of rats to x-radiation, but different in that the daily exposures required only a few minutes.<sup>5</sup> No evidence of "stimulation" was apparent in the peripheral blood of these animals. In those instances in which an effect occurred, it was invariably a reduction in the number of circulating cells.

No deliberate or well controlled human experiments have been done which are comparable in chronicity to these animal experiments. The experiments of Low-Beer and Stone,<sup>6</sup> and Nickson, Cantril, and Jacobson,<sup>7</sup> however, in which human subjects were exposed up to a total dose of 300r total body given in divided doses of from 5 to 20r (x-ray) produced a general reduction in the various hematologic constituents of the peripheral blood. In several cases studied by Low-Beer and Stone,<sup>6</sup> however, an absolute monocytosis became apparent reaching in several instances more than 50 per cent of the circulating leukocytes. It has not occurred in the human cases we have studied nor has it been seen in the many animal experiments referred to above.

## 2. EFFECTS OF FOCALIZED IRRADIATION

In mice exposed to large amounts of  $\beta$  rays from plaques containing  $P^{32}$  there was some focalized damage in several organs adjacent to the skin. Changes were very marked in ovaries and testes. But the changes which are of interest in this communication occurred in the spleens of a few of the animals. On the dorsal surface of the spleen there was a zone of severe radiation damage. This zone gradually merged into normal spleen, the gradation consisting of progressively diminishing damage without any evidence of a zone of hyperplasia of undamaged cells. Since the  $\beta$  rays have only a limited range of penetration, it would be expected that near the periphery of their range the small amount of radiation would evoke a stimulating effect if such an effect does occur.

This lack of a stimulating effect at the periphery of an area of focalized radiation is also characteristic of the changes which occur after localized deposition of radioactive isotopes. When these isotopes accumulate in given areas in radiosens-

sitive organs, they produce localized areas of radiation damage—much more extensive with  $\beta$  emitters than with  $\alpha$  emitters because of the longer range of the former. The penetration of alpha rays is only a fraction of a millimeter in tissue whereas the  $\beta$  ray penetrates several millimeters. In or near none of these areas of focalized damage is there any evidence of cellular stimulation as evidenced by hyperplasia on normal cells.

With the exception of the highly diffusible  $\text{Na}^{24}$ , the majority of the isotopes studied tended to localize in bone and most of them also accumulated in the red pulp of the spleen and in other organs. This resulted in a marked hypoplasia and aplasia of the bone marrow. As a consequence, most of the spleens of these animals showed a great increase in ectopic myelopoiesis over the normal. This obviously is in compensation for the aplastic bone marrow. In no instance was a primary stimulation produced in the blood forming tissue which reflected an increase in circulating cells. Compensatory increases were, however, noted in a few instances. The radioisotope,  $\text{Sr}^{89}$ , is fairly generally distributed to blood forming tissue within the first few days after its parenteral administration. It produces a reduction almost immediately in the nucleated cells of the peripheral blood. It translocates, however, to bone and, therefore, exerts its major effect on the bone and bone marrow. The lymphocytes, however, remain depressed for long periods even though normal lymphopoiesis is resumed in the lymph nodes and spleen. This phenomenon occurs with other isotopes which are not localized more than temporarily in lymphatic tissue.

In some of the rats to which  $\text{Sr}^{89}$  was administered osteogenic sarcomas developed eight to ten months later. This might be considered as a "stimulation."

#### DISCUSSION

It is clear from the histologic examination of our animals that there is no evidence of a primary stimulation of hematopoiesis by small amounts of irradiation from either generalized external, or focalized internal sources. The changes in the cells of the circulating blood after irradiation point to the same conclusion, although the analysis is complicated by factors of mobilization and localization in the various parts of the circulatory system. The heterophil increase which follows rather large acute exposures to penetrating radiations is, according to Isaacs,<sup>5</sup> a hastened maturation of heterophil precursors and thus a "stimulation."

The mechanism of the spectacular monocytosis described by Low-Beer and Stone<sup>7</sup> in certain of the humans exposed to divided doses of total body x-irradiation is not clear. Since it occurred only in individuals with degenerative arthritis, it may be a response associated with the underlying disease process.

Irradiation of the hematopoietic organs as reflected in changes in the peripheral blood is often stated in the literature to produce stimulating effects. The articles most frequently referred to in this connection are those of Murphy and his co-workers.<sup>9</sup> Actually these authors stated, 'We have further noted that by one small dose of x-ray we could obtain in a certain proportion of animals a stimulation of the lymphoid elements, preceded by a comparatively short period in which the lymphocytes were below normal.'

Other statements are found in the literature which have affected the conception that x-rays produce a stimulating effect on blood forming tissue. These effects, however, have been described after large doses of penetrating x-irradiation. An increase in the erythrocytes per cu mm and hemoglobin in Gm per 100 ml,<sup>10</sup> an increase in the platelets,<sup>11</sup> <sup>12</sup> reticulocytes,<sup>13</sup> <sup>14</sup> and polymorphonuclear neutrophils<sup>15</sup> have been described by a number of authors. These and other papers on this subject, however, generally dealt with inadequate numbers of control and experimental animals and, therefore, deny statistical validation. Reports are also found in the literature describing the findings in the peripheral blood of man after chronic exposure to ionizing radiation. These reports stress the significance of a lymphocytosis,<sup>16</sup> monocytosis,<sup>17</sup> eosinophilia,<sup>18</sup> <sup>19</sup> leukopenia,<sup>19</sup> <sup>20</sup> <sup>21</sup> anemia either normocytic or macrocytic<sup>22</sup> <sup>23</sup>. These reports have tended to instill a sense of security in those working with radiation because of the implication that peripheral blood findings could be used as an indication of effect on a given individual even with radiations in the 'tolerance range' (0.1 r total body exposure per day). There is no work to substantiate this. As significant as many of these studies have been, the re-evaluation of these concepts is indicated at this time because of studies conducted on the Plutonium Project in the past few years.

Our findings and interpretations are in accord with the general conclusions of Czepa<sup>24</sup> and Packard.<sup>25</sup> As the latter points out: "The evidence now at hand points to the conclusion that radiations do not directly stimulate normal activities of the cells, their primary effect is always an injury from which the cell may recover perfectly. But the degeneration products may temporarily quicken the tempo of some normal processes, such as protoplasmic streaming and mitosis, an acceleration which is followed by a retardation and often by very obvious injury. Such reaction is secondary, and is not true stimulation in the sense in which the term is used in radiological literature."

### CONCLUSIONS

Extensive studies with acute and chronic application of externally originating ionizing radiations and internally deposited radioisotopes have failed to reveal evidence in the blood forming tissues and peripheral blood of a primary stimulation of hematopoiesis. However, secondary or compensatory increases in certain of the cellular constituents of the peripheral blood were seen and were invariably preceded by a reduction. The initial leukocytosis (heterophil increase) which occurs in the first twenty-four hours after acute exposure to externally originating irradiations is probably a reaction to injury mediated through a mobilization rather than a new formation of blood cells. The abortive rise in heterophils, lymphocytes, and reticulocytes is likewise probably a result of frank injury.

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# THE EFFECT OF RADIATION ON HEMOPOIESIS IS THERE AN INDIRECT EFFECT?

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## I. INTRODUCTION

SINCE the discovery of the x-ray by Roentgen, medical science has evinced great interest in elucidating its mode of action. Today, the problem is by no means completely solved. Certain facts, particularly those relating to the results obtained when tissues are subjected to direct radiation, are well established and widely accepted. For example, the aplasia of bone marrow resulting from direct widespread radiation in large doses must be regarded as beyond dispute.

However, there are other observations both of a clinical and experimental nature which are poorly understood and highly controversial. These relate particularly to changes in tissues far removed from the direct site of radiation and have led to the postulation of an 'indirect' action of x-rays. For example, many roentgenologists have privately observed, and others have publicly reported, marked involution of lymph nodes involved in a lymphomatous or metastatic process when radiation was given to other areas sufficiently remote as to have precluded any direct exposure of the former. Equally well known is the marked reduction in the blood count of leukemias treated by x-ray and the leukopenia which develops infrequently in similarly treated nonleukemic individuals, even when supposedly very small areas of marrow are subjected to the effects of direct radiation. These effects, at least in superficial appearance, are not unlike those well established for direct radiation. The result has been a welter of interesting but confusing literature which will presently be reviewed.

It is with this possibility of an "indirect" action of roentgen radiation that this report is concerned. The concept of 'indirect' action requires further definition. It is inconceivable in an organism whose every cell is bathed in a continuous, circulating fluid medium, and which is interlaced with an intricate network of nervous tissue, that such an agent as x-ray, known to be capable of creating extensive destruction of certain cells, should be without an indirect effect. It is well established that there are indirect effects from a thermal burn or mechanical crush. Yet in the case of crush injuries, at least, the only action directly attributable to the causative agent ceases at the time the trauma is discontinued. What occurs in the organism after that must be regarded as strictly nonspecific, even though these events may be of a highly serious nature at times. We, therefore,

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have limited our approach to an effort to demonstrate indirect effects *peculiar* to radiation. The pertinent question is whether or not radiation results in the formation of specific substances (not merely those liberated by nonspecific cellular injury or death) which when transported to distant areas produce a characteristic indirect effect.

If such distant indirect effects exist, it seems reasonable that the agents mediating them will in all probability be transmitted to their site of action by way of the circulation. The experimental approach has been, therefore, to arrange for the direct passage of a very large volume of blood for a period of several hours from a radiated to a normal animal, and then subsequently to observe the normal animal for evidence of changes which might be interpreted as indirect radiation effects. Cross circulation by way of carotid to carotid anastomoses satisfies the concept of this approach. Parabiosis, in which small circulatory connections undoubtedly exist between the two animals, was felt to be disadvantageous in that only small amounts of blood cross circulate per unit of time. The methods and technics employed will be described in detail below. Important corollary principles are that the radiated animal must receive a relatively large acute dose of radiation in order that any indirect effect present may be made more manifest, and that sufficient experiments must be done to cover all time intervals up to at least a few days after radiation of the one animal. Significant changes in the leukocyte and lymphocyte picture in the normal animal following cross circulation have been selected as the criteria of an indirect effect in these investigations. These criteria were selected because (1) these elements have been shown to be among the most sensitive indicators of damage by radiation, and (2) changes in these elements and their precursors have been repeatedly mentioned by those attributing indirect effects to radiation.

## 2. REVIEW OF THE LITERATURE

An appreciable amount of medical literature has accumulated concerning the 'indirect' effects of x-rays. Particularly in the early 1900's many contributions to the subject were making their appearance, the German investigators being especially active in this field. Much of this early literature is mutually contradictory and largely of historical interest. Dosage of radiation administered was in most instances not accurately known, and the quality of radiation employed varied widely from investigator to investigator. Roentgenology was in its infancy and technical factors poorly understood and poorly controlled. Nevertheless, the field is not one that readily lends itself to experimental investigation, and even with vastly increased knowledge and improved technical control, the same general arguments prevailing soon after the turn of the century are still being debated in the literature of the last twenty years.

Broadly these investigations can be grouped into four main types: (1) The demonstration of, or the failure to demonstrate specific toxins (most frequently 'leukotoxins') in the serum of patients or experimental animals exposed to radiation. Both *in vivo* and *in vitro* studies of this nature are reported. (2) The demonstration of, or the failure to demonstrate significant histologic changes in tissues

ordinarily sensitive to roentgen radiation following radiation of some site remote from the tissues studied (3) The clinical demonstration of significant involution of susceptible tissues far removed from the point of application of direct radiation (4) A group of miscellaneous investigations chiefly concerned with demonstration of a wide variety of biochemical abnormalities occurring after radiation, and purported to be the mediators of an indirect effect

Efforts to demonstrate a specific toxin developed as a result of radiation have been particularly numerous Linser and Helber<sup>26</sup> (quoted by Capps and Smith) found that a leukotoxin was produced in the blood of organisms exposed to x-ray This leukotoxin when injected into other animals destroyed the circulating leukocytes, and when added to animal exudates containing leukocytes caused loss of motion and degeneration of the cells Curschmann and Gaupp<sup>7</sup> in 1905 injected into rabbits serum taken before and after radiation therapy from a patient with lymphatic leukemia A few hours after injection the authors observed leukopenia not present in control animals This, they concluded, was due to the development of a specific leukotoxin which could also be demonstrated as capable of destroying human leukocytes in vitro They presented evidence suggesting that such a leukotoxin was inactivated by heat (60 C for one half hour) The following year Klieneberger and Ziepitz<sup>20</sup> were unable in their investigations to demonstrate any consistently toxic action of radiated serum on leukocytes in vitro either as to cellular disintegration or as to influence on amoeboid activity In addition no constant leukopenic effect was observed after injection of radiated serum into rabbits The authors concluded no toxin could be demonstrated as a result of radiation Milchner and Wolff<sup>29</sup> observed some decrease in leukocytes in animals after the injection of 5 cc of serum from a radiated animal of the same species They also noted some degree of leukopenia when material from a radiated spleen was injected, while injections of normal spleen resulted in leukocytosis However, they felt that their results were not of sufficiently concrete nature to be strong evidence for the presence of a leukotoxin, particularly in view of wide, physiologic variability in the leukocyte counts of the experimental animals used Benjamin<sup>1</sup> and co-workers after radiating the ears of rabbits with large doses while shielding the remainder of the animal found leukocytosis and lymphopenia occurring, but the blood picture returned to normal within twenty-four hours They claimed to have found increased choline formation in animals after intense radiation, and to have noted that this corresponded to the period of leukocytosis The investigations of Capps and Smith<sup>6</sup> were interpreted as favoring the concept of a leukotoxin These authors found leukopenia to result from the injection of a few cc of radiated serum from one animal into the abdominal wall of another This serum was also said to cause abnormal destruction of leukocytes in hanging drop preparations With this view Harris<sup>15</sup> concurred, though presenting no experimental evidence of his own

In 1918 Dorn<sup>10</sup> reported that testicular and ovarian atrophy and leukopenia of considerable duration resulted from the injection of enzitol, a borate of choline The author felt that the effects of radiation were exactly reproduced by this substance and that this offered strong support to the concept of choline as the toxic cause of radiation effects Walterhofer<sup>31</sup> likewise considered indirect effects of

radiation to result from some toxin, possibly choline- In 1922 Billings<sup>5</sup> in discussing his experiences with the treatment of leukemia by roentgen ray, stated that the application of radiation to any cutaneous surface resulted in the appearance of a leukolytic substance in the blood. The serum of a treated patient dissolved white blood cells in vitro, while the serum of an untreated patient had no such effect. In the same year, Murphy, Liu and Sturm<sup>31</sup> were forced to conclude that their experiments failed to show any evidence of the presence of a lymphotoxin, even when exposures large enough to effect almost complete destruction of lymphoid tissue were employed. Zacherl<sup>56</sup> four years later, as a result of experiments with radiation of single and parabiotic rats, felt that some toxic substance capable of causing sickness and death of the animals must have been produced. Szilárd<sup>49</sup> in 1927 could demonstrate no autolytic effect on leukocytes in the serum of radiated leukemics, nor could any such autolysin be demonstrated by complement fixation tests. He could find no increase in choline in the blood or urine of leukemics under x-ray therapy. This author postulated none the less that perhaps electrons circulating in the blood exerted deleterious effects on distant sensitive cells. Strumia's<sup>47, 48</sup> studies led him to conclude sweepingly in 1929 that 'radium emanations, as such, are carried by the blood or that they act through the production of leukotoxins, which are produced by the direct effect of the radium emanations upon the white cells of the blood in superficial vessels, or perhaps upon fixed cells of the reticulo-endothelial system.' He further stated, 'The direct action of radiation upon hemopoietic foci is altogether unimportant. In all cases, the blood acts as a carrier of radiations, either as such or modified, to the hemopoietic foci where its main action takes place.' Woenckhaus<sup>55</sup> felt that leukopenia observed in normal rats after injection of serum from strongly radiated rats was due to products of proteolysis in the injected serum. However, he also found that when one member of a parabiotic pair was radiated and the other shielded, a *leukocytosis* and not *leukopenia* occurred in the non-radiated parabiont. In 1932, Zwerg<sup>58</sup> heavily radiated skin flaps in small laboratory animals. The remainder of the animal was shielded, yet if the pedicles connecting the skin flap to the animals remained intact, they uniformly died in two to nine days, and exhibited varying degrees of leukopenia and lymphopenia. The latter, however, were not of the order of magnitude uniformly seen in animals who have received enough generalized radiation to result in death. Zwerg concluded that the effect of roentgen rays on the white blood cell picture is not a direct action on the blood forming organs and probably not on the circulating blood. Rather, he believed, the decomposition of cells gives rise to toxins which in turn damage the hemopoietic system.

Macht<sup>27, 28</sup> in more recent years has attacked the problem by phytopharmacologic methods, examining blood and serum obtained both before and after radiation from a variety of animals. His method, briefly, consists of studying the growth of roots of *Lupinus albus* seedlings in standard physiological solutions as compared with the growth of similar seedlings in similar solutions to which blood or serum had been added. This investigator concluded that there is a toxic substance present in radiated but not in normal serum, and that it reaches its maximum titer twenty-four to forty-eight hours after treatment, disappearing in a few days.

Osgood<sup>16</sup> in 1942 conducted *in vitro* experiments designed to determine if indirect radiation effects on cells of bone marrow could be demonstrated. He set up four human marrow cultures identical in every respect except one received no radiation, in one both cells and medium (35 per cent human serum and 65 per cent balanced salt solution) were radiated, in one *only* the medium was radiated, and in one *only* the cells were radiated. Quantitative hematologic studies indicated no indirect action of radiation on marrow cells suspended in the radiated medium.

A number of histologic studies have also been made in which tissue remote from the direct site of radiation has been examined for evidence of indirect damage. Nakahara and Murphy<sup>11</sup> found identical changes in both deep and superficial lymphoid tissue following small doses of soft x-ray. Since 96.8 per cent of the rays did not penetrate beyond a depth of 1 cm. it was felt these changes in deep tissue could not be the result of direct action by the rays. Jolly and Ferroux<sup>19</sup> in histologic studies made on lymphatic structures outside the field of direct radiation were of the opinion that there was grave doubt that the slight changes noted were due to an indirect, toxic action of radiation. Rather it was their inclination to attribute such slight changes as might exist to diffused or secondary radiation. Akaiwa and Takeshima<sup>1</sup> exposed popliteal lymph nodes of rabbits to various amounts of radiation, leaving one side as a nonradiated control. The changes in lymphoid tissue on both sides were noted at a variety of intervals after radiation. The authors concluded that in the control nodes histologic changes identical with those in the radiated nodes occurred, but that the reaction was much less intense and much slower on the nonradiated side. In 1940, Hsu and Ma<sup>16</sup> radiated one femur only of rats with doses varying from 1000r over a four day period to 5000r over a forty day period. The day after the course of radiation was complete animals were sacrificed and the bone marrow of radiated and nonradiated femurs, the submaxillary lymph nodes, and the spleen of each were examined. In the nonradiated hemopoietic tissues changes of a *hyperplastic* nature interpreted as compensatory, were noted. Le Blond and Segal<sup>25-42</sup> found large doses of roentgen rays produced secondary changes in well shielded organs far distant from the point of impact. These consisted of constant thymus and generalized lymphatic atrophy and adrenal hypertrophy and frequently of fatty infiltration of the liver and ulcerations of the stomach. Such lesions were considered part of a general intoxication following radiation and were felt to be similar to those described by Selye as occurring in the nonspecific "alarm reaction" developing after the application of a wide variety of injurious stimuli. It is interesting (as has been noted with other noxious agents capable of eliciting an "alarm reaction") that thymus and lymphatic atrophy was suppressed by adrenalectomy but the gastric lesions and general lethal effects of the rays increased. Barnes and Furth<sup>8</sup> in 1943 using single and parabiotic mice reported an extensive series of investigations. These authors examined bone marrow, lymph nodes, and spleen in unexposed areas of mice radiated with anywhere from 400r to 7000r. Some histologic changes were observed, but these were slight and regarded as probably the result of products of damaged tissue transported by way of the circulation. The degree of change depended on the dose of radiation and probably on the volume of tissue radiated. The authors observed similar histologic

changes in mice burned under anesthesia with the actual cautery over the chest and abdomen, and in the lymph nodes of two mice dying of iodoacetic acid poisoning. In addition, the effect on the hemopoietic tissue and lymph nodes of normal mice whose parabionts had been subjected to radiation was studied. Histologic changes similar to but much less extensive than those found in the radiated animal were observed in the tissues of the nonradiated parabiont. These were considered non-specific. Densted,<sup>9</sup> likewise in 1943, reported on a large number of examinations of bone marrow and peripheral blood in patients being treated with x-ray. Control specimens were obtained before treatment. The majority of the patients were suffering from malignant disease, and x-ray dosages, while variable, were for the most part cancericidal in magnitude. Marrow specimens were obtained a considerable distance away from the sites exposed to direct radiation. Twenty-eight patients developed no granulocytopenia (below 3000 granulocytes per cubic millimeter). In these there was no abnormality of the nonradiated marrow either as regards total quantity of cells or differential values. In 20 patients the granulocytes of the peripheral blood fell below 2300 per cubic millimeter. In these cases, the cellularity of the nonradiated marrow remained normal, but adult polymorphonuclear leukocytes in the marrow decreased from an average of 16 per cent in control groups to 6.5 per cent in granulocytopenic groups. The promyelocytes were unquestionably increased. For these reasons, the author concluded there is in certain patients some degree of maturation inhibition in myelopoiesis, and that this is probably due to some toxic or anaphylactic factor. He considers this to be an indirect effect of roentgen and radium rays on hematopoiesis and suggests it may occur more readily in patients who are in poor condition.

While many roentgenologists have observed phenomena suggestive of an indirect radiation effect, relatively few have seen fit to publish these clinical observations. However Langer<sup>23</sup> has stated that he had observed indirect radiation effects on several occasions. He cites the case of a boy treated with x-ray for verruca simplex of both hands. Although only one hand was treated the warts gradually disappeared from the untreated hand. He refers also to a case reported by Baensch in 1922 where, in a patient with primary carcinoma of the breast, regional nodes disappeared even though shielded with lead rubber during radiation. Scott<sup>41</sup> has reported to have observed frequently in cases where large areas were radiated the disappearance of lymphosarcomatous glands in regions receiving no radiation. In one case, he states, this was manifest within half an hour after therapy.

In addition to the above, a miscellany of diverse mechanisms have been postulated as the mediators of indirect radiation effects. In 1905, Musser and Edsall<sup>32</sup> concluded that the effect of x-ray is not direct but is dependent on a reaction of the body subsequent to exposure. They felt that the increased calalysis observed after radiation was a response in the form of an increased fermentative process, and concluded that favorable results to x-ray therapy could occur only when the body was capable of such response. Demiéville<sup>8</sup> concluded that an indirect effect of radiation, probably conditioned through decomposition products, was exhibited in the blood-forming organs. Petersen and Saelhof<sup>38</sup> in 1921 demonstrated increased serum titers of several enzymes after radiation and speculated whether some remote effects of

radiation may not be due to enzyme mobilization. Hussey<sup>17</sup> found an uncompensated alkali excess in rabbits exposed to x-rays and suggested alteration in acid-base balance may be an important factor in their action. Opitz<sup>18</sup> suggested that toxins produced by x-rays may be agglutinated by tumor cells, thus impairing tumor growth. Rahm and Kooser<sup>19</sup> assumed chemical changes are initiated in the dead interstitial substances of the body and that these may be responsible for the end effects seen. V. Pannwitz<sup>20</sup> observed acidosis followed by slowly increasing alkalosis which persisted for several days after radiation. Kluge and Zwerg<sup>21</sup> have suggested that mobilization of hormones, particularly those of the hypophysis, are important in producing the observed effects of radiation. Selye<sup>46</sup> in an extensive review of general adaptation phenomena has pointed out that many of the metabolic changes described after radiation such as hyperglycemia, decrease in blood cholesterol, increase in ketone bodies in the blood, elevation of the NPN, and disturbances in acid-base balance are seen with equal frequency as a response to many injurious stimuli such as traumatic shock, exposure to cold, burns, drugs, and solar rays. This author feels such things represent nonspecific systemic phenomena elicited by injurious stimuli to which the organism is qualitatively or quantitatively not adapted. Very recently Dougherty and White<sup>13</sup> have proposed that roentgen radiation exerts both a direct and indirect effect on lymphocytes and that the indirect action can be explained on the bases of the increased pituitary—adrenal cortical activity caused by radiation.

It is readily apparent that the literature weighs heavily on neither side of the question.

### 3 EXPERIMENTAL TECHNIC AND METHODS

The experimental animal employed was the cat and the chief experimental device cross circulation by way of carotid to carotid anastomoses. In the twenty-six successful cross circulation experiments which form the basis of this report, a normal animal was in each instance cross circulated with a radiated animal. The latter received its radiation either during the actual time of cross circulation or at varying intervals prior to its establishment. A control group in which normal cats were cross circulated had already been established in this laboratory in connection with experiments previously reported.<sup>24</sup>

The technic of establishing cross circulation was as follows. The animals were anesthetized with nembutal administered intraperitoneally. The operative site was prepared, and the carotid artery of each on one side isolated and dissected free. A small rubber covered clamp was placed at the proximal and distal ends of the freed vessel and the artery divided midway between the two clamps. Both segments were then washed free of blood with isotonic citrated saline solution. In each animal, the proximal arterial segment was cannulated by threading it through a Monel cannula, everting the cut end of the vessel over the outside of the cannula, and tying with fine 5-0 catgut. The two animals were next brought together in such a way that the proximal arterial segment of each was directly adjacent to the distal segment of the other. They were conveniently supported in this position by the use of small sandbags. A bed for the anastomoses was readily formed by sutur-

ing together the strap muscles of the two animals. Following this, the distal arterial segments were drawn over the outside of the cannulated proximal segments and tied about the cannula. Clamps were then released and cross circulation allowed to function from two to ten hours. The cross anastomoses thus established afforded a continuous endothelial pathway by which the blood from each cat supplied one side of the head of its partner and vice versa. Figure 1 diagrams the method of anastomosis.

The volume of blood traversing an anastomosis of this type, will, if uncompensated, result in the exsanguination of an animal within a few minutes. At the conclusion of the period of cross circulation the patency of each connection was always tested *in situ* by reapplying the clamps, dividing the anastomoses *distal* to each cannula, and releasing the clamps for a brief instant.

Prior to an experiment, control hematologic studies consisting of hemoglobin determinations and a total and differential white blood cell count were made. Only animals which were clinically well and which showed no gross hematologic abnormalities were used. During the period of cross circulation leukocyte counts

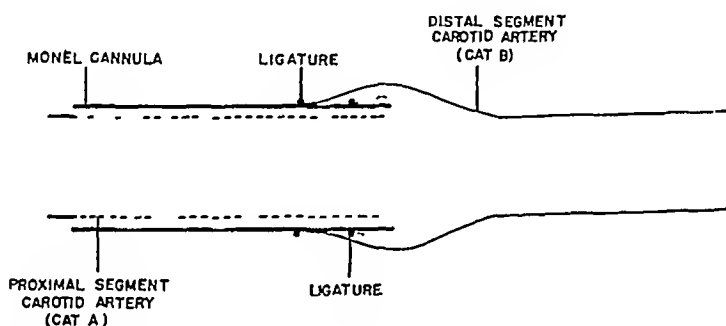


FIG. 1. Diagram of end to end carotid to carotid anastomosis of the type employed in these experiments.

and smears were made at approximately hourly intervals. In each instance blood was obtained from the marginal ear vein. The hematologic follow-up of the non-radiated animal consisted of a minimum of daily (except Sunday) total and differential white blood cell counts for a period of two weeks and similar studies made every other day for a period of approximately an additional two weeks. All counts were made with Bureau of Standards certified pipets. All blood smears were made by the coverslip method and stained with Wright's stain.

The radiation to which one member of each cross-circulated pair was subjected was kept constant and subject to the following factors:

Total Dosage	1500r whole body radiation
Voltage	k v p 250
Milliamperage	15
Target Distance	22 inches to center of cat
Filter	Aluminum parabolic plus $\frac{1}{2}$ mm copper
Half Value Layer	2.1 mm copper
Rate of Administration	Approximately 25 r per minute with slight variation

This amount of radiation invariably results in the death of cats within four to seven days

In the majority of experiments, one animal was radiated at some interval prior to cross circulation. In seven experiments, however, cross circulation was first established and then one animal radiated while the other was shielded. Shielding was accomplished by means of an appropriately shaped wooden frame covered with one-fourth inch of lead plate. An ionization chamber placed under the frame recorded the small amount of scatter radiation which escaped the shielding. This varied in different experiments from a minimum of 20 r to a maximum of 43 r.

It was customary to administer isotonic saline subcutaneously to both animals during cross circulation and at its conclusion. In some instances, penicillin in saline was also administered.

#### 4 PRESENTATION OF DATA

In all, twenty-six successful cross circulation experiments were performed. Of these, all the normal animals except one were followed hematologically for a period of approximately twenty-eight days after return to their own circulation. Cat number 153 had to be sacrificed about two weeks after cross circulation because of a wound infection. With one exception, each cross circulated team consisted of one radiated and one nonradiated member. The experiment involving normal cat number thirty-six who was cross connected in series with two radiated animals simultaneously constituted this exception. In such a circuit, blood from the normal animal passed to one radiated animal, which in turn was connected to a second radiated animal, which likewise in turn supplied blood to the normal cat.

The data pertinent to these experiments are especially suited for presentation in graphic and tabular form and this has been the mode of presentation selected.

Figure 2 shows in graphic form the duration of cross circulation and the interval after radiation of one member of the team that cross circulation was established. It can readily be seen that in the majority of experiments the duration of cross circulation was in the neighborhood of eight hours. A few animals were cross circulated as long as ten hours, one for as little as two hours and seven minutes. The group of animals in which radiation of one partner took place at the time the cross circulation was functioning were connected for approximately three to five hours. More experiments were performed during or shortly after the radiation of one member because it was felt that logically the most profound effects on the normal partner could be expected under these circumstances. Nevertheless, all the time intervals after radiation up to eighty-two hours were covered.

Table 1 presents detailed hematologic data from each experiment and indicates the total leukocyte and absolute lymphocyte count of each normal animal before and at varying intervals after cross circulation with a radiated partner. The intervals selected were done so arbitrarily with an eye to conserving space without sacrificing a comprehensive and representative presentation of the data. In addition, in the experiments in which one animal was shielded and the other radiated during the period of cross circulation, the amount of radiation recorded by the



ionization chamber under the shield has been indicated. In every experiment of this type a small amount of scatter radiation escaped the shielding.

Tables 2 and 3 give the means and standard deviations for total lymphocyte and leukocyte counts respectively of the normal animals for the same time intervals before and after cross circulation as given for the individual animals in table 1. The figures are divided into three groups: those applying to the shielded group of normals, which were cross circulated at the time of their partner's radiation, those applying to the non-shielded group which were cross circulated with a previously radiated partner, and those applying compositely to the group as a whole. It will be noted that the number of animals for which data are presented varies somewhat on different days in the tables. This is due to the fact that counts were made but six

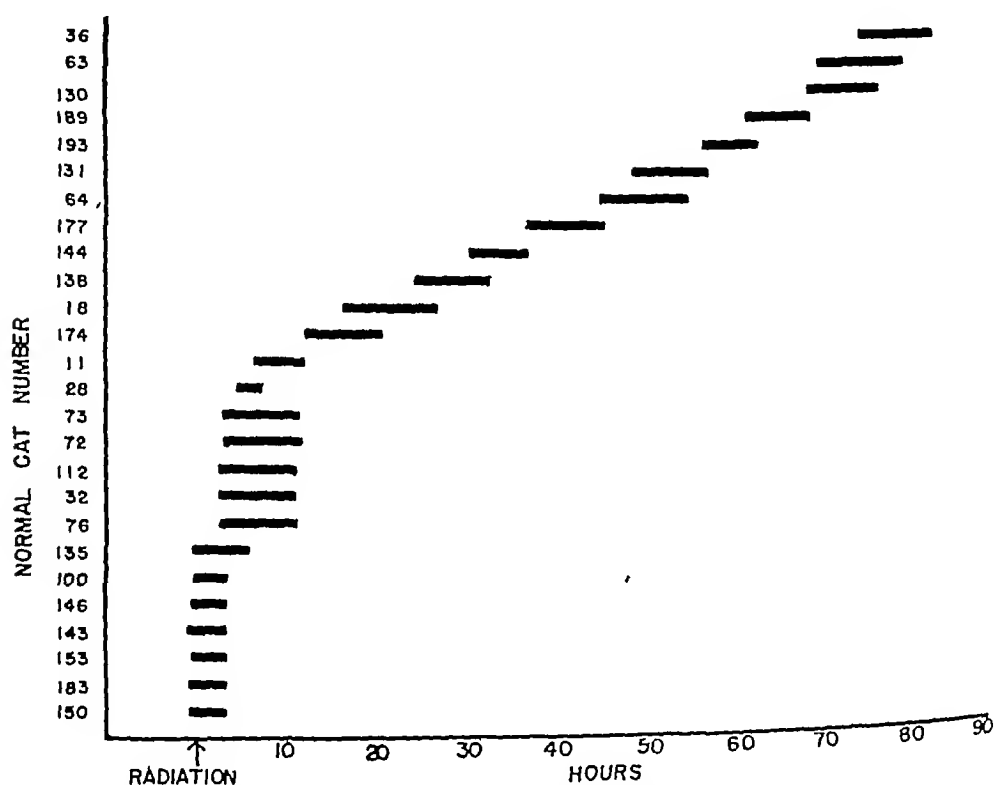


FIG. 2. Each black line indicates the duration in hours of cross circulation in a single experiment. The time interval after radiation of one member that cross circulation was established is also indicated.

days a week so that at any specified interval after cross circulation it is possible that counts are not available for every member of the group.

Figure 3 indicates graphically a composite picture of the average leukocyte and lymphocyte counts of the entire group during the period of follow-up. In addition, the same data on the seven shielded animals are graphed separately, both because these animals unavoidably received small amounts of radiation, and because this type of experiment was considered the most critical of the group.

## 5 RESULTS

Analysis of the data indicates few or no detectable hematologic abnormalities in the normal cat at any time after its cross circulation with a radiated animal.

TABLE 1—Total Leukocyte and Absolute Lymphocyte Count Per Cubic Millimeter of Twenty six Normal Cats Following Cross Circulation with Radiated Animals

Cat No.	Duration of circ in hrs	Amt of Radiation	Prior to circ	First After circ	1 day	2 days	3 days	5 days	7 days	10-11 days	14-15 days	21-22 days	End of Exp
100†	3 0	33r	23 000* 2 415†	64 000 2 560	41,000 1 230	35 200 1,232	19 100 1,054	23 100 1 848	29,600 3,625	21,900 2 080	29,900 2 691	27 300 3 003	13 500 1,620
135†	6 0	20r	14 200* 3 408†		31 500 945	25,300 1,265		13 500 1,135	19,800 1,683	27 700 2 353	21 800 3 161	12,300 3,874	9,400 1 692
143†	3 8	43r	21 200* 3,180†	18 600 837	22 600 2 260	21 200 1,696	15,000 900		22,000 2,420	14,800 1,628	13,800 3,588	17,700 2,301	34,700 3 470
146†	3 7	28r	16 400* 4 410†	19,000 950	26,700 934	34 700 1,561	35 100 2 281		18,300 1,921	10,800 854	25 200 1,897	45 900 2 065	35 500 1 242
150†	3 6	35r	12 100* 1 815†	55 000 2 750	17 100 171	16 200 243	15 000 975	26 000 780	19,300 1,447	23,000 805	12 800 344	8,200 1 129	25 400 386
154†	3 3	23r	5 600* 1 708†	19 000 3 783	24 500 1 592	15 400 2 464	25 200 2,006	36 300 3 085	19,700 3 152	31 700 4 438			
183†	3 7	24r	7 800* 1 560†	30 600 1 683	30 500 2 135	11 300 1,243	11,400 1,710		12,200 854	8 400 1,008	12,600 1,280	6 900 1 173	7 500 900
28	2 1	none	23 800* 7 378†	54 500 5 450	68 800 8,256	59 500 7 735	30,250 9 362	34 300 6 174	29 600 5 032	44,900 6 285	21,700 4 774	14 300 6 435	12 000 4,200
11	5 0	none	15 200* 4 408†	36 100 2 888	30 100 3 311	31 900 9 689	33 400 5 678	24 500 4 900	21 500 3,655	14 800 4 440	8 900 2 670	4 200 2 184	8 400 3,870
18	10 0	none	8 800* 2 904†	28 900 1 445	29 000 2 610	28,300 849	23,100 2 541		15,900 3,498	12 400 2 108	7 800 3 354	8 000 4,080	11 100 4 551
64	9 5	none	14 000* 2 660†	28 300 849	13 800 1 932	24 500 3,675	16 200 3 240	16 500 2 640	21 000 2,310	13,700 4 521	28 500 7 980	12 700 4,318	14,600 5,256
63	10 0	none	20 800* 5 824†	16 100 1 449	21 000 2 100	16 000 1,920	15 600 3 432	20 900 5 852	22 000 3,520	21,200 3 604	19 100 4 584	25,500 8 415	20 200 5,454
36	8 8	none	34 700* 8 328†	14 000 1 820	22 500 3 150	35 700 2 409		29 100 5 820	23 900 3 107	35 000 3 500	26 100 3,654	15 600 4 524	19 900 2,587
73	8 0	none	13 200* 3 564†	51 500 1 030	54 100 1 082	31 400 7,556	28,200 5 358	24 600 4 183	27 000 3 510	25 700 1 542	21 000 2 310	16 100 2 415	11 000 2,860
72	8 2	none	13 600* 3,128†	50 800 2 032	34 300 3 087	30 900 4,635	29 400 4 998	39 200 3 920	22 300 5 352	22,100 5,525	16 700 5 010	10 600 1 908	22 700 2 270
32	8 0	none	13 700* 3,151†	44 900 2,245	34 200 2 394	32 100 4 173	16,400 5 084		19,000 4 465	10 500 1 470	18,700 4 488	15 000 2 850	11,400 3 534
12	8 2	none	13 800* 3 312†		26,300 1 052		25,800 3 354	14 500 1 450	17,300 3 114	44 600 3 122	42,700 2 989	32 800 2,624	17,500 5 425
76	8 2	none	18 700* 2 244†		30,700 2 19	43 000 1 720	25 700 2 136	19 000 1 710	16,600 3 652	25 600 2 560	20,100 2 010	14 300 2 860	13,200 1 188
31	8 1	none	12 100* 3 267†		20 600 2 884	21 700 1 953	13 200 3 168	14 300 1 716	10 600 2 120	19 000 5 130	10 300 2 266	10 300 2 266	10 200 2 856
30	8 0	none	16 300* 2 608†		44 760 2 682	30 930 2 72		30 560 2 440	26 400 1 848	25 000 000	20 700 2 691	14 400 2 592	20 000 1 800
38	7 7	none	10 500* 2 625†		27 460 822		17 260 2 236	21 800 3 488		12 800 896	10 500 2 366	10 700 1 923	16 700 1 336

TABLE 1—Continued

Cat No	Duration of x circ in hrs	Amt of Radiation	Prior to x circ	First After x circ	1 day	2 days	3 days	5 days	7 days	10-11 days	14-15 days	21-22 days	End of Exp
44	6 3	none	11,900* 3,332†		14,300 1 287		13,100 2,358	23,600 2,124	20,300 3,248	11,400 2 052	9 300 1,023	13,700 959	20,500 3 483
74	8 0	none	10,900* 1,090†	27 000 0	29,000 0		13,400 1,742	8,500 1,105	11,300 1,582	10 100 1,111	6,300 630	5,700 798	12 600 1,764
177	8 2	none	5 900* 2 478†		11,200 1,680		14,700 2 352	18,200 2,730	11,700 702	15,100 2 265	17,200 3 268	15 500 3,255	15,200 3,648
189	7 0	none	6 000* 2 040†	22,800 0	17 800 1 780	14,500 1 740	14,500 2,030	23,600 1 888	15,600 624	18,900 1,512	17 300 2 768	24,700 1,976	15 300 1 939
193	5 8	none	10,200* 3 162†		15 500 3 410	25 900 7,770	12,000 3,120	15 000 2,250	14,800 2,220	10,100 2,626	13,800 4,830	17,700 4,248	12 400 3,720

\* = Total Leukocyte count

† = Absolute Lymphocyte count

‡ = Shielded animals

TABLE 2—Means and Standard Deviations for Absolute Lymphocyte Counts of Normal Cats Following Cross Circulation with Radiated Animals

	Shielded Group			Non shielded Group			Entire Group		
	N	Mean	S D	N	Mean	S D	N	Mean	S D
Prior to x-circ	7	2642 3	1063 1	19	3552 8	1798 2	26	3307 7	1664 0
First after x-circ	6	2093 8	1145 2	11	1746 2	1515 0	17	1868 9	1368 9
1 day after x-circ	7	1323 9	734 8	19	2403 6	1697 3	26	2112 9	1562 8
2 days after x-circ	7	1386 3	665 1	14	4183 3	2892 4	21	3251 0	2719 5
3 days after x-circ	6	1804 3	1159 5	17	3658 2	1928 2	23	3174 6	1924
5 days after x-circ	4	1712 0	1017 4	17	3199 4	1657 7	21	2916 1	1646 8
7 days after x-circ	7	2157 4	974 4	18	2975 5	1320 4	25	2746 4	1270 0
10-11 days after x-circ	7	1882 3	1279 2	19	3066 8	1579 1	26	2747 9	1573 3
14-15 days after x-circ	6	2160 2	1221 8	19	3351 3	1675 1	25	3065 4	1638 6
21-22 days after x-circ	6	2257 5	1063 7	19	3191 3	1849 6	25	2967 2	1721 5
End of Experiment	6	1551 7	1056 8	19	3252 3	1346 4	25	2844 1	1463 5

N = Number of animals on which counts are available at the time specified in column 1

The average leukocyte count for the entire normal group and for the critical group in which cross circulation was established at the time of radiation of their partner remains throughout the entire approximately twenty-eight day period of follow-up actually somewhat higher than the pre-cross circulation control level. In not even a single animal did evidence of significant leukopenia develop at any time during the experiment — this despite the fact animals received for a period of several hours the full volume output of the carotid artery from a radiated partner. As would be expected, well marked leukocytosis was present in many instances for a few days after the operative procedure. Its duration varied rather widely from

TABLE 3—Means and Standard Deviations for Total Leukocyte Counts of Normal Cats Following Cross Circulation with Radiated Animals

	N	Shielded Group		N	Non shielded Group		N	Entire Group	
		Mean	S D		Mean	S D		Mean	S D
Prior to $\lambda$ -circ	7	14328 6	6461 4	19	14426 3	6658 5	26	14400 0	6476 4
First after $\lambda$ -circ	6	34433 3	20133 3	11	34081 8	14440 3	17	34205 9	16032 1
1 day after $\lambda$ -circ	7	27700 0	7624 3	19	28700 0	14484 4	26	28430 8	12853 4
2 days after $\lambda$ -circ	7	22757 1	9440 5	14	30450 0	11216 7	21	27885 7	11060 1
3 days after $\lambda$ -circ	6	20133 3	8714 5	17	20185 3	7254 9	23	20171 7	7452 4
5 days after $\lambda$ -circ	4	24725 0	9385 2	17	22241 2	7860 7	21	22714 3	7977 7
7 days after $\lambda$ -circ	7	20042 9	4988 9	18	19266 7	5510 8	25	19484 0	5278 3
10-11 days after $\lambda$ -circ	7	19757 1	8704 6	19	20678 9	10798 4	26	20430 8	10115 0
14-15 days after $\lambda$ -circ	6	19383 3	7323 0	19	17736 8	8665 8	25	18132 0	8246 8
21-22 days after $\lambda$ -circ	6	19716 7	14826 0	19	14831 6	6872 4	25	16004 0	9260 2
End of Experiment	6	21000 0	12572 7	19	14994 7	4153 6	25	16436 0	7261 1

N = Number of animals on which counts are available at the time specified in column 1

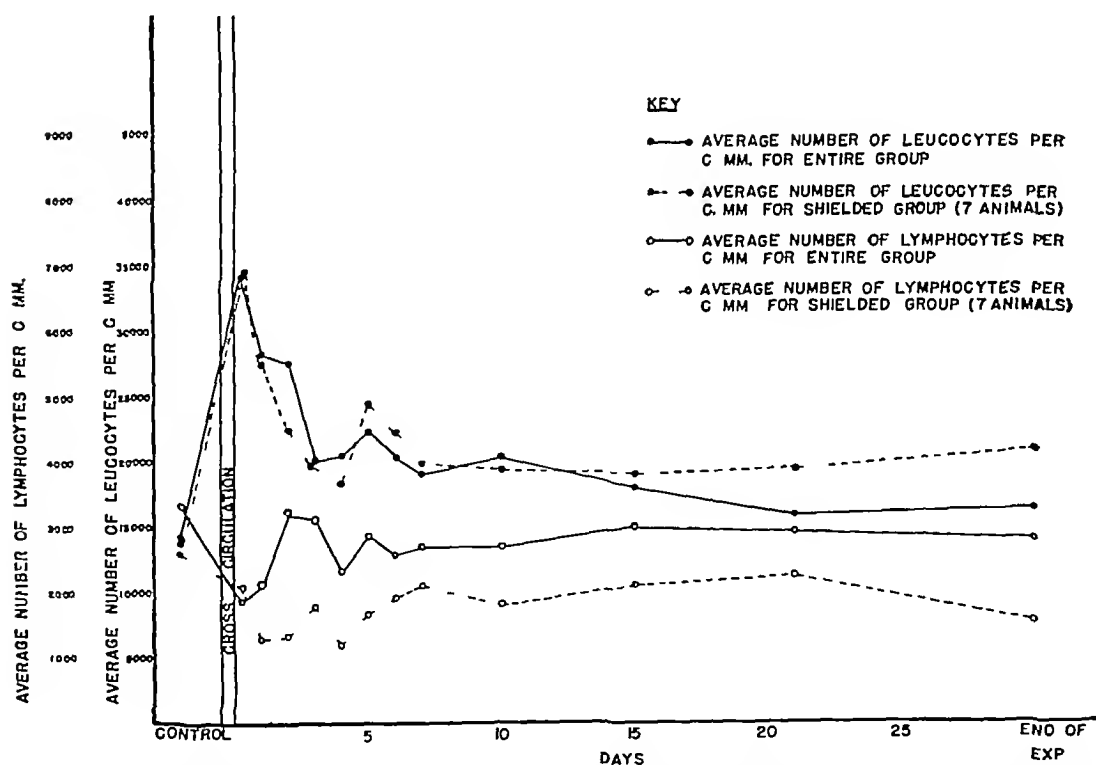


FIG 3 Average leukocyte and lymphocyte counts per cubic millimeter of the whole group of animals for the entire period of the experiment are indicated. The average leukocyte and lymphocyte counts for the critical shielded group are graphed separately.

animal to animal. Of some interest is the very striking degree of leukocytosis developing at the time of cross circulation in certain animals whose partners had received their radiation at the time of or shortly before cross circulation. Whether these more striking instances of leukocytosis are due to the passage of leukocytotic

metabolites resulting from tissue breakdown as a result of radiation cannot be said with certainty. The leukocyte counts in question were markedly in excess of those developed in a normal control group, however.

Individual lymphocyte values found in normal animals after cross circulation show a moderate amount of variation. As would be expected during the period of marked leukocytosis during the few days immediately following cross circulation, a drop in the absolute lymphocyte count was noted. Lymphopenia is a well known companion of neutrophilic leukocytosis and in this instance, it was presumably accentuated by the fact some animals had been cross circulated with partners who had no circulating lymphocytes at all. To be sure, the average lymphocyte level for the entire group remained throughout slightly lower than that found before cross circulation and this trend was slightly more apparent in the critical shielded groups as a whole. In view of the physiologic variations in lymphocyte counts, in view of the persistence of some *increase* in the average total leukocyte counts throughout the experiment, and more particularly in view of the fact that the reduction in lymphocyte count was relatively slight and not at all of the order of magnitude associated with direct radiation, there is grave question that any significance can be attached at all to this finding. At no time did the average per cent of lymphocytes fall below 4.9 per cent of the leukocytes present even in the shielded group, and this figure was obtained only for the first day after radiation. In all other instances it was appreciably higher than this.

## 6 DISCUSSION

It is apparent that under the conditions of our experiments, no specific "indirect" effect of radiation has been demonstrated. It is also felt that if significant indirect effects peculiar to radiation are dependent upon the circulation, they should have been readily demonstrated by the experimental setup employed. However, it cannot be denied that had it been possible to maintain cross circulation for much longer periods—say forty-eight or seventy-two hours instead of eight to ten hours—indirect effects too slight for demonstration during the shorter period might have become manifest. The possibility of an indirect radiation effect mediated solely by the lymphatics has also been considered but discarded as highly unlikely. Any lymphatic disseminated agent would in all probability soon reach the venous and arterial tree and would be expected to manifest itself in the blood. Further, if in some devious manner it should be removed from lymph before reaching the blood, indirect effects of radiation would exist purely in the regional lymphatic drainage area of that portion of the body directly radiated. The existence of such a purely localized regional indirect radiation effect is supported by no evidence and, in fact, is contrary to whatever evidence there is suggestive that such indirect effects exist.

It is also apparent that there is no satisfactory evidence for a *characteristic* indirect radiation effect presented in the medical literature. Such evidence as is presented is of dubious character and refuted for the most part by contrary results obtained in similar types of experiments by other investigators. Nor has there been more than a desultory attempt on the part of investigators of indirect radiation effects to

differentiate body reactions common to a variety of damaging stimuli from those specific to radiation. This is a fundamental differentiation, particularly in view of Selye's now well supported observations that an identical, nonspecific systemic reaction (at times severe enough to result in death) occurs as a response to many unrelated injurious stimuli. Still, it is undoubted that leukopenia develops in a certain number of individuals treated with roentgen radiation even when the amount of marrow directly exposed to radiation is thought to be minimal. It is also highly likely that involution of and histologic changes in normal and abnormal lymphoid tissue remote from the site of direct radiation as observed by many roentgenologists and research workers is real and not apparent. It would seem wise to examine these considerations more closely to see if it is necessary to assume they are peculiar to radiation and if they can be accurately described as *specific* attributes of radiation per se.

Kornblum<sup>22</sup> and his associates in an extensive series of clinical observations concluded that therapeutic radiation tended to lower the leukocyte count. However, in approximately one-half of the patients studied there was no decrease in the number of neutrophils and in the great majority of the remaining cases depression was of slight degree. In but 7 cases out of 120 were the neutrophils reduced below 2000 per cubic millimeter. The effect of therapeutic radiation on the lymphocytes was more striking, a definite decrease in their numbers being the rule. In most cases the drop in count was relatively slight but in some instances it was very pronounced. It is thus seen that leukopenia of appreciable degree is an inconstant feature of radiation therapy applied to areas where there are no or minimal amounts of hematopoietic tissue. While the area treated, the volume of tissue radiated and the total amount of radiation are, in our opinion, all of importance as regards the production of leukopenia, yet it is impossible to predict accurately in most instances of local radiation over essentially non-hematopoietic areas which individuals will and which will not develop leukopenia. On the other hand, one can always anticipate the development of leukopenia when radiation is given over any appreciable amount of hematopoietic tissue such as in total body irradiation. Further, leukopenia associated with total body irradiation is more marked than when no or minimal amounts of hematopoietic tissue are radiated. Thus radiation over large amounts of active bone marrow can be expected to produce marked leukopenia, whereas radiation over non-blood forming areas produces mild, if any, leukopenia. Still another difference between the results of radiation over hematopoietic and nonhematopoietic areas is to be found in the morphological appearance of the bone marrow. With radiation directly over the bone marrow, hypocellularity and/or aplasia result, whereas the marrow shows normal cellularity or it may even be hyperplastic when other tissues are exposed and it is excluded.

Three possible explanations for these differences arise. First, they may be only quantitative, the so-called indirect effect actually being a direct effect resulting from inclusion of larger amounts of hematopoietic tissue in the field of radiation than generally considered to be the case. It should be noted in this connection that the exact amount of hematopoietic tissue exposed to radiation is not known in

many instances where an indirect effect is said to occur. It may well be that the amount of blood producing tissue radiated in these cases is sufficient to reduce the output of blood cells to a point where their normal level cannot be maintained. In particular, one must consider the possibility that in debilitated patients the reserve for production of blood cells may be distinctly lower than in the normal. If this is true, the inclusion of even small amounts of hematopoietic tissue in the radiated field in patients receiving radiation therapy would be of much greater significance than ordinarily considered since most of such patients are suffering from malignancy or some other serious disorder which is associated with debilitation. As a further indication that the effect may be due to inclusion of hematopoietic tissue in the radiated field is the well established fact that the leukopenia practically never results from radiation of the head in which case radiation can be given without inclusion of more than minimal amounts of active bone marrow. Second, a specific indirect effect of radiation may exist, i. e., some substance or substances may be produced directly and characteristically by radiation and then transmitted to parts of the individual that were not exposed to radiation. Our experiments have failed to reveal such a situation. Third, a nonspecific effect of radiation may occur, that is, radiation over a local area may produce certain nonspecific changes in the tissues exposed. As a result of these nonspecific effects histological changes may develop in some unexposed tissues and leukopenia possibly be produced also. It is again possible here that the nonspecific effects may be greater in a debilitated than in a normal individual. Our experiments have failed to show the presence of any nonspecific substance capable of producing leukopenia. We have not examined the tissues histologically but feel that the evidence in the literature is sufficient to justify the assumption that these nonspecific histologic changes did occur at least in the normal animals which were being cross circulated at the time their partner was irradiated.

Substantial involution of lymphoid tissue not itself directly injured has been frequently observed as a nonspecific response to a wide variety of injurious agents. Bardeen<sup>2</sup> in studying visceral changes occurring in patients dying of superficial burns noted striking histologic changes of a degenerative nature in lymphoid tissues and commented on the similarity of these changes to those found experimentally after injection of ricin or diphtheria toxin. These widespread alterations in all the lymphoid organs of the body following burns have been amply confirmed by other investigators.<sup>52, 37, 3</sup> Similar changes and transient leukopenia and lymphopenia have been observed in mice subjected to dry heat in nonfatal exposures.<sup>30, 33</sup> Selye<sup>43, 44, 45</sup> in what he has chosen to term the "alarm reaction," has described striking involutionary and degenerative changes in all the lymphatic organs as a nonspecific response to a variety of insults (cold, heat, surgical shock, drugs). Nonspecific damage apparently may result in atrophy of the spleen beginning in the center of the Malpighian corpuscles, marked loss of weight of thymus and lymph nodes, and, at times even complete disappearance of germinal centers in the latter. Zechwer<sup>57</sup> noted similar changes after injuring subcutaneous tissues by injection of formalin. It is interesting that this involution of lymphoid structures does not occur in the adrenalectomized animal—particularly in the light

of recent investigations suggesting that lymphoid structures and possibly the lymphocytes may in some way be affected by the administration of adrenal cortical hormone<sup>18 11 12 46 53 54 40 14</sup> In view of the above observations, it is possible that x-ray is merely one of many injurious agents capable of producing generalized involution of lymphoid tissue as a nonspecific response to local injury This, of course, is not to be confused with local involution and degeneration of lymphoid tissue directly exposed to radiation

The question of whether or not a specific indirect effect of radiation exists must be regarded as unsettled It is our feeling that leukopenia, when it results from radiation, is probably due at least in part to direct exposure of some of the hematopoietic tissue Other factors which may be operative are the general physical status of the patient, nutritional disturbances resulting from the effects of radiation to the gastrointestinal tract, general radiation sickness, and the amount and degree of total body irradiation resulting from scattered radiation We also feel that morphologic changes occur in certain nonexposed tissues (e g , lymph nodes, and thymus) We are not convinced, however, that these nonspecific changes produce leukopenia

## 7 SUMMARY

1 The general aim of the investigations here reported has been to obtain evidence for or against "indirect" radiation effects

2 To this end, twenty-six successful cross circulation experiments (carotid to carotid anastomoses) have been performed between normal cats and radiated cats

3 Cross circulation was established in most instances at some specified time interval after the radiation of one partner All intervals up to eighty-two hours after radiation of one partner were covered

4 In seven experiments cross circulation was established and then one animal radiated while the other was shielded These were considered the most critical experiments of the group

5 Detailed data on leukocyte and lymphocyte counts in the normal animals obtained during an approximately twenty-eight day period of follow-up are presented

6 These data are not considered to support the thesis of indirect effects peculiar to radiation A trend toward slightly lowered absolute lymphocyte counts in normal animals after cross circulation was not considered significant, and in no instance did leukopenia develop in the normal animal

7 The literature is reviewed and discussed

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## NORMAL BLOOD COUNTS IN DIFFERENT SEASONS

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**B**LOOD counts, like various other routine examinations, were introduced into clinical medicine a rather long time before their physiologic variations and the ranges of their normal values had been finally determined. Hence, all statements of leukocyte counts and differential counts in normal individuals suffer from want of uniformity. Sex and age, and perhaps even race must be considered. Exercise and excitement bring about a conspicuous increase of the number of leukocytes,<sup>1</sup> whereas variations due to static changes are less significant.<sup>2</sup> The alleged "digestive leukocytosis" has not been confirmed by modern investigations.<sup>3</sup> Further diurnal rhythms of the neutrophils have been noticed,<sup>4</sup> presenting a climax in the afternoon and shortly after midnight. Likewise, rhythmical variations have been observed showing maxima and minima within about one hour.<sup>5</sup> According to Friedlander and Wiedemer,<sup>6</sup> the number of reticulocytes rises conspicuously during the months of spring, dropping to a low in the autumn, whereas, inversely, the hemoglobin and the number of erythrocytes show the lowest values during spring. Further, these authors have stated that heliotherapy is followed by a rise in the number of reticulocytes, they therefore suggest that seasonal variations in the reticulocyte count must be due to changes in the intensity of sunlight.

In order to decide whether results of routine blood examinations are subject to seasonal variations worth mentioning, we have carried out examinations four times during one year (in the months January, March, June and October) on 40 male and 29 female healthy students of medicine determining the hemoglobin per cent (Haldane), the number of erythrocytes and the number of reticulocytes (per one thousand erythrocytes), and the sedimentation rate, counting, in addition, white cells and eosinophils (Dunger), and making differential counts of 300 cells on cover glass smears. The examinations were performed in the morning on venous blood, after half an hour of muscular rest, all the tests being examined by the same person.

The hemoglobin value for both sexes drops to its lowest in October, the difference being statistically significant, reduction of the average figures is about 10 per cent, equally, the color index seems to be lowest in October, the number of erythrocytes, however, being constant. The highest reticulocyte numbers are found for both sexes in the most sunny periods of the year, the deviation being, however, not significant, whereas the sedimentation rate is found to be lowest in June and highest in October for both sexes. The white cell count and that of their fractions present great variations although the averages vary but slightly and insignificantly, it must, however, be kept in mind that the choice of the four months of examination has been arbitrary, and contingent maxima might fall outside these periods.

The results present a conspicuous variation especially from one individual to another at a given time of the year, whereas variations in the same person examined

From the "Finsen-Laboratorium" and the University Institute of Pathological Anatomy, Copenhagen

TABLE 1 --The maximal and minimal findings, and the average values of blood counts made on 40 males and 29 females at different times of the year

	January	March	June	October
Average number for 40 males				
Hemoglobin (per cent)				
Erythrocytes (millions)	100 (112 - 90)	97 (106 - 87)	99 (110 - 87)	89 (99 - 78)
Color index	5 06 (5 96- 4 57)	4 94 (5 50- 4 41)	5 00 (5 58- 4 50)	4 96 (5 56- 4 28)
Reticulocytes	0 99 (1 07- 0 88)	0 98 (1 07- 0 87)	0 99 (1 08- 0 91)	0 90 (0 97- 0 75)
Sedimentation rate	4 2 (15 - 1)	4 2 (10 - 1)	5 8 (18 - 1)	4 6 (10 - 1)
White cells	2 6 (6 - 1)	2 5 (10 - 1)	2 2 (6 - 1)	3 0 (5 - 1)
Neutrophils	5830 (9440 - 3500)	5700 (117,000 - 3320)	6050 (12,000 - 3400)	5900 (9080 - 3480)
Eosinophils	3150 (6600 - 1320)	3160 (6520 - 1300)	3580 (5760 - 1400)	3280 (6850 - 1090)
Lymphocytes	215 (480 - 72)	194 (450 - 90)	204 (510 - 51)	235 (480 - 60)
Monocytes	1900 (2610 - 995)	1800 (2520 - 1200)	1690 (2600 - 980)	1790 (2600 - 1260)
	550 (970 - 240)	520 (1010 - 120)	530 (725 - 360)	560 (690 - 250)
Average numbers for 29 females				
Hemoglobin (per cent)				
Erythrocytes (millions)	86 (100 - 70)	84 (97 - 95)	84 (95 - 74)	77 (89 - 72)
Color index	4 41 (5 10- 3 76)	4 40 (5 30- 3 82)	4 32 (5 01- 3 75)	4 24 (4 75- 3 78)
Reticulocytes	0 97 (1 07- 0 79)	0 95 (1 07- 0 84)	0 97 (1 06- 0 90)	0 91 (0 99- 0 80)
Sedimentation rate	4 5 (13 - 1)	3 4 (10 - 1)	5 0 (11 - 1)	4 7 (10 - 1)
White cells	5 2 (11 - 2)	4 5 (8 - 1)	3 6 (10 - 1)	5 5 (10 - 3)
Neutrophils	5620 (8120 - 3640)	5430 (7440 - 3520)	5380 (7600 - 3940)	5570 (7480 - 4140)
Eosinophils	3210 (6100 - 1530)	3170 (5380 - 1690)	3220 (5410 - 1920)	3130 (4530 - 2050)
Lymphocytes	162 (300 - 30)	176 (422 - 61)	179 (389 - 53)	169 (560 - 50)
Monocytes	1740 (2770 - 1040)	1580 (2680 - 1100)	1490 (2080 - 1140)	1740 (2430 - 1270)
	510 (760 - 250)	480 (880 - 270)	450 (820 - 240)	510 (1070 - 250)

at different seasons are less pronounced. This indicates that physiologically some individuals have a high, and others a low leukocyte level. The averages for the 40 males and 29 females examined at different seasons will be seen in table 1. The seasonal variations which have been observed are less pronounced than the individual variation at a fixed time of the year, in other words the seasonal variation is without significance to the estimation of the results achieved by common blood counts. The present figures, thus, yield a basis of evaluation of the normal average values for adult men and women. The results may be seen from table 2, showing further the maximal and minimal findings, the marked standard deviation is illustrated in figure 1. Since each leukocyte group may present relatively high per cent values even with a low leukocyte total, and vice versa, there can be ascribed no special importance to the cell percentage which must be considered only as an aid to

TABLE 2.—*The maximal and minimal findings, and the average values of blood counts made on 40 males and 29 females at different times of the year*

	Men			Women		
Hemoglobin (per cent)	96	(78 - 112)		82	(70 - 100)	
Erythrocytes (millions)	4.99	(4.28 - 5.96)		4.35	(3.75 - 5.30)	
Color index	0.97	(0.75 - 1.08)		0.95	(0.79 - 1.07)	
Reticulocytes	4.7	(1 - 10)		4.4	(1 - 13)	
Sedimentation rate	2.6	(1 - 10)		4.6	(1 - 11)	
White cells	5870	(3320 - 12,000)		5500	(3520 - 8120) <sup>1</sup>	
Neutrophils	3310	(1090 - 6850)		3190	(1530 - 5820)	
Eosinophils	210	(51 - 510)		170	(30 - 422)	
Monocytes	540	(117 - 1014)		490	(240 - 1070)	
Lymphocytes	1800	(980 - 2610)		1640	(1040 - 2770)	
Neutrophils (per cent)	55.0	(38.7 - 74.5)		57.4	(42.4 - 75.7)	
Eosinophils (per cent)	3.4	(0 - 10.0)		2.9	(0.5 - 8.0)	
Monocytes (per cent)	9.4	(4.0 - 14.0)		8.9	(5.3 - 14.3)	
Lymphocytes (per cent)	31.6	(11.3 - 47.0)		30.0	(15.0 - 45.0)	

the estimation of the absolute number of the different white blood cells. A lymphocyte per cent of about 50, thus, may very well be normal, but this is conclusive only when the absolute number is to be found within the ranges of 1000-3000. The lines drawn by Naegeli and Schilling,<sup>5, 8</sup> 20-25 per cent and 21-35 per cent respectively, therefore, must be considered too narrow, whereas the absolute figures come up very well to the statements given by, e. g., Boerner,<sup>1</sup> Schilling,<sup>8</sup> and Saltzmann.<sup>7</sup> It is, however, worth mentioning that the upper normal range of monocytes and eosinophils is 1000 and 500 respectively. According to the above examinations, the normal values for adult men and women are

White cells	3,000-10,000 per cu. mm. of blood
Neutrophils	1,000-6,000
Eosinophils	50-500
Monocytes	100-1,000
Lymphocytes	1,000-3,000

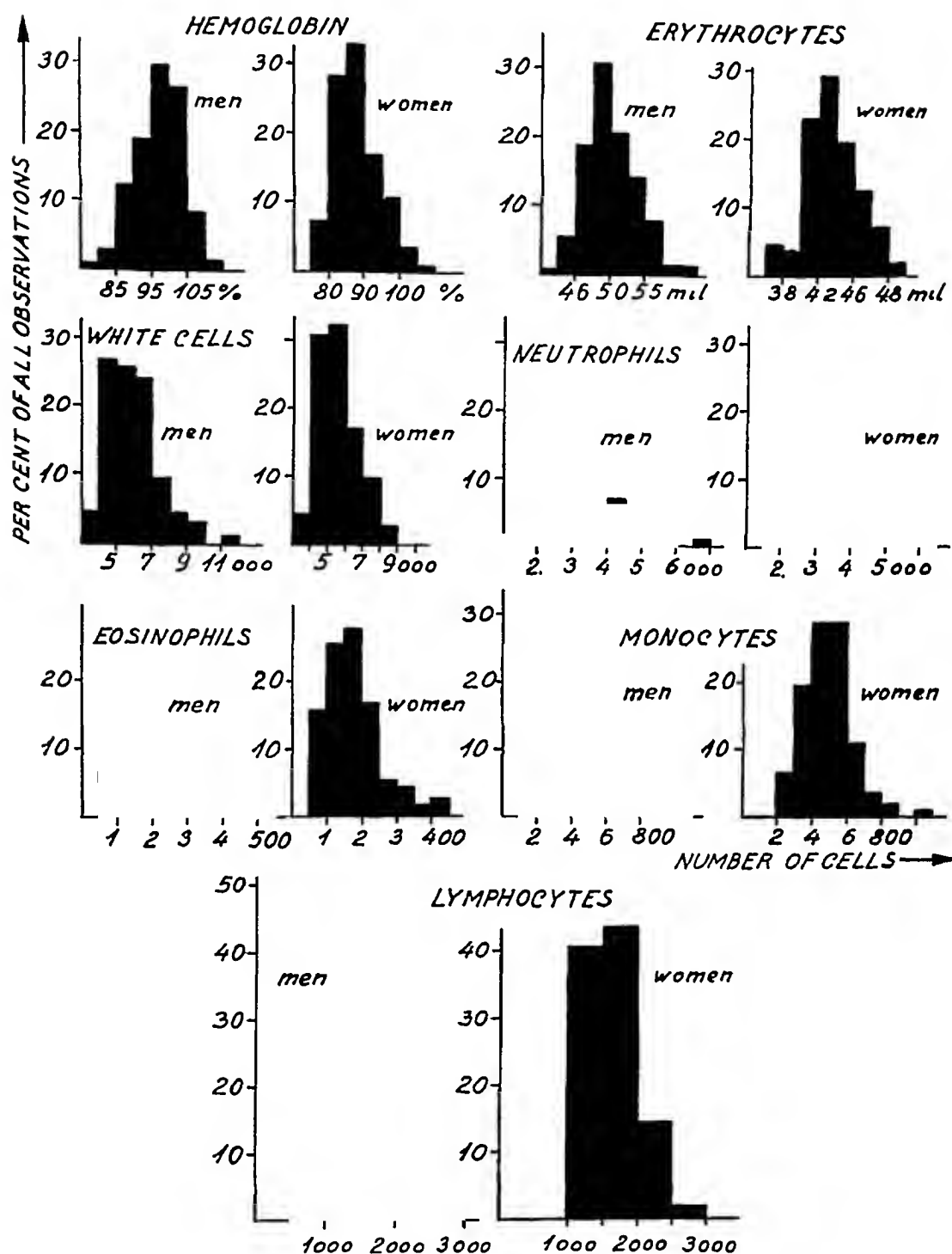


FIG 1 SHOWING BLOOD COUNTS FOR 40 MEN AND 29 WOMEN EXAMINED  
FOUR TIMES DURING ONE YEAR

The diagrams show the percentage of all observations occurring within the different intervals

#### SUMMARY

Report is given of examinations carried out on 40 healthy males and 29 healthy females in the months of January, March, June and October, with determination of

the hemoglobin, the number of erythrocytes and reticulocytes, the sedimentation rate, the white cell and differential counts. The hemoglobin value appears to be lowest in October, the number of reticulocytes highest in June, the sedimentation rate lowest in June. The remaining figures present no exact seasonal variations. Some individuals have a high leukocyte level, others a low one. Normal values are illustrated.

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# BLOOD

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### EXOGENOUS HEMOCHROMATOSIS RESULTING FROM BLOOD TRANSFUSIONS

By STEVEN O SCHWARTZ, M D , AND SUNOLL A BLUMENTHAL, M D

HEMOCHROMATOSIS was first described by Hartman and Chausser in 1882 as "bronze diabetes" because of the association of skin pigmentation with diabetes mellitus. Von Recklinghausen in 1889 showed that the pigmentation of the skin and viscera was due to deposits of hemosiderin and hemofuscin. Hemochromatosis was considered quite rare, being clinically diagnosed only three times in 160,000 admissions to the Johns Hopkins Hospital, and on postmortem examination only four times in 5,000 autopsied cases at the Bellevue Hospital. There were less than 100 cases in the literature before 1920. By 1935, Sheldon<sup>43</sup> was able to collect 311 acceptable cases and at the time of the last complete review of the literature in 1941<sup>4</sup> there were 436, indicating either a growing incidence or, more likely, a keener awareness and consequently more frequent recognition of the disease.

Classically, hemochromatosis occurs in the male, the ratio being 295 males to 16 females in Sheldon's series. It is practically unknown before the age of 20 and has its peak incidence between 45 and 55 years. The fully developed disease presents (a) an enlarged liver (caused by a hypertrophic cirrhosis), (b) a bronze pigmentation of the skin, which usually has a peculiar slaty blue or metallic appearance,<sup>13</sup> (c) diabetes mellitus of a severe type, and (d) a form of sexual hypoplasia characterized by impotence and an alteration of the hair distribution.<sup>27</sup> However, one or more of these features may be absent.<sup>24</sup> Pathologic characteristics are (1) the pigment deposition, (2) the fibrotic changes, (3) the cellular degeneration in certain of the parenchymatous organs, this being the least prominent. The disease is inevitably fatal, death resulting either from the diabetes or from cirrhosis of the liver. The life expectancy in Butt and Wilder's<sup>6</sup> series varied from one month to 13 years, averaging one and one-half years from the time the diagnosis was made. Sheldon puts the figure considerably higher, stating that the average terminal period is 18½ years.

Anemia is very rare in hemochromatosis, the average blood values being 4.1 million red cells and 80 per cent hemoglobin.

The two kinds of pigment found in hemochromatosis are hemosiderin and hemofuscin. Hemosiderin is an iron containing pigment, deep yellow in color, the particles varying in size from fine granules to larger masses which may be visible to

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the naked eye Hemosiderin contains about 55 per cent iron Its reactions are not typical for ferric iron and there is no reaction for ferrous iron Cook<sup>9</sup> considers it to be some form of colloidal ferric oxide physically combined with an organic substrate Hemosiderin is the predominating pigment of hemochromatosis and has an extreme affinity for gland cells of both external and internal secretion The greatest amounts are found in the liver and pancreas, but there is no secreting gland in the body that may not be affected Striking exceptions to involvement are the kidneys and the germinal epithelium of the testes The former has only a moderate degree of pigmentation, confined in most cases to the convoluted tubules of the second order, while the latter usually escapes altogether Striated muscle is, but smooth muscle is not affected In at least 90 per cent of the cases, the heart muscle is pigmented, often by large amounts Considerable quantities of pigment are found in the reticulo-endothelial system They occur in the Kupffer cells of the liver, the alveolar endothelium of the lung, and in the spleen In structures where there is much pigment in the tissue cells, there is almost always pigmentation of the connective tissue as well, this being especially so in the liver and pancreas

Hemofuscin is a dark, almost black, pigment which contains no iron It contains about 3.7 per cent sulphur and is probably related to melanin In hemochromatosis it occurs to a slight extent in the epithelial cells of the glandular organs, but with more frequency in the connective tissue, especially that of the spleen There is nearly always a large quantity in the heart muscle, but its site of election is the smooth muscle of the genital and alimentary tracts, and the smooth muscle of arteries

An invariable feature of the disease is cirrhosis of the liver, usually of the hypertrophic type The pancreas is also constantly affected, though usually less severely In contrast to the changes which occur in the liver, pigmentation and cirrhosis do not always go hand in hand in other organs and there may be an advanced interstitial pancreatitis with only a small amount of hemosiderin The spleen nearly always shows some fibrosis as do the heart, thyroid and salivary glands The fibrosis, both in the liver and pancreas, has usually been considered the *result* of irritation produced by the iron pigment Sheldon, however, concluded that the two processes, namely hemosiderosis and fibrosis, are independent of each other Herbut and Tamaki<sup>21</sup> agree with this view and emphasize the fact that fibrosis occurs almost exclusively in the liver, pancreas and spleen, and is not found in other organs in spite of the fact that hemosiderosis may be very severe in them They believe that the excess of fibrous tissue in both pancreas and spleen is due to portal hypertension Degenerative changes in the parenchymatous cells do not occupy a prominent place apart from the direct mechanical damage to the cells by the surrounding fibrous tissue Even where there is no pigment it is common to see collections of degenerated cells surrounded by thick rings of fibrous tissue

Chemical investigation reveals a great increase of iron in the various organs which may be 50 to 100 times normal in the liver, pancreas and salivary glands The total amount of iron in the body has been estimated at from 20 to 58 grams, of which as much as 38 grams has been found in the liver Spectrographic studies have revealed an increase of calcium in the tissues paralleling the increase in iron, ex-

ceptions being the lung, trachea and urinary bladder, which have less calcium than the control tissues, but have a marked increase of potassium. An increase of copper has been noted in most tissues,<sup>39</sup> exceptions being the suprarenals and jejunum which have normal values, and the kidney which has less than normal.

### *Relation of Hemochromatosis to Various Etiologic Factors*

Soper<sup>44</sup> reported a typical case of hemochromatosis in a metal worker who was in contact with large amounts of copper, lead and zinc. Creed<sup>10</sup> had a man who drank wine from a barrel lined with copper, while Gray<sup>18</sup> described the case of a copper miner. Lawrence<sup>29</sup> reported on the familial incidence of the disease in a family of nine siblings. Of the 4 males, 2 had typical hemochromatosis, a third had a borderline case, while the fourth was unaffected. Vitamin A deficiency with defective intestinal mucosa has also been postulated as possibly responsible.<sup>46</sup>

*Iron metabolism in hemochromatosis* Although studies made by Keilin<sup>26</sup> in 1926 on the tissues of a case of hemochromatosis did not reveal any obvious disturbance in the intracellular metabolism of iron, it is felt that the iron cytochrome is undoubtedly subject to replacement in health as a result of the normal wear and tear of the tissues, and may be left in a form which the cells are unable to excrete when due for replacement. This would also account for the increased copper, since Elvehjem<sup>12</sup> has shown that copper is necessary for the formation of cytochrome. In normal individuals, iron is taken in the diet in amounts of from 8 to 10 milligrams daily, partially absorbed in the duodenum, and excreted in minimal amounts through the large bowel and the kidneys. Iron is stored in the liver and only a slight trace is excreted in the bile. The body normally contains less than 5 grams of iron, more than half of which is in the hemoglobin. In hemochromatosis, the body excretes some iron in the stools, but none in the urine or bile. The iron accumulates in the body,<sup>1</sup> as much as 58 grams having been reported.

The dietary intake of the average individual would provide enough iron for normal blood formation with an excess of from 25 to 50 grams by the age of 45 or 50 if the disease were congenital. If this were so, one might even expect to find cases with a familial incidence. Such have indeed been reported,<sup>29</sup> but for some reason have attracted little attention.

Howard and Stevens,<sup>22</sup> McClure,<sup>34</sup> Fowler and Barer,<sup>14</sup> and Dry<sup>11</sup> have all shown that iron absorption is no greater in hemochromatosis than in normal patients. Fowler and Barer however, had an early case which did demonstrate increased absorption over a short period of study. Marble and Smith, in 1939,<sup>33</sup> gave iron to a patient with hemochromatosis for a twelve day period. Absorption was 1.8 milligrams per day, the same as in a normal control. Sachs, Levine and Griffith<sup>41</sup> showed the average normal whole blood iron to be 50 micrograms, and copper to be 0.132 micrograms per 100 cc. In their 3 cases of hemochromatosis the values for whole blood iron ran from 37 to 44 micrograms and copper from 0.130 to 0.156 micrograms. They thought that the low iron values were due either to the slight anemias which were found in all 3 cases or that the increase in the deposition of iron in the tissues tended to reduce the quantity of circulating iron.

*Experimental Hemochromatosis* Muir and Dunn, in 1915,<sup>36</sup> produced a rapid he-

molysis in rabbits by injecting an immune body intravenously. They demonstrated that most of the iron resulting from destruction of the red cells was deposited in the liver, spleen, and kidney on the fourth and fifth days. After the ninth day these organs contained little over the normal amount of iron. Rous and Oliver, in 1918,<sup>11</sup> injected rabbits with rabbit blood for several months without producing hemochromatosis. They did, however, demonstrate hemosiderin deposits in the liver. Mallory, in 1921,<sup>32</sup> gave rabbits copper acetate for as long as eleven and a half months. He produced pigmentation, similar to hemofuscin, and cirrhosis in three rabbits. He believed that hemofuscin is changed to hemosiderin in some way and, after saturating the liver, is deposited in the pancreas, adrenal, thyroid, spleen, heart, skin and stomach. Polson, in 1929,<sup>37</sup> gave rabbits dialyzed iron intravenously for three months and then sacrificed them one week to fourteen months later. He demonstrated that the iron moved from the lungs to the liver via the spleen. No evidence of cirrhosis or hemochromatosis was noted in the liver. Cappell, in 1930,<sup>7</sup> gave colloidal iron to rabbits and showed that the iron was taken up by the reticulo-endothelial system, later entered into loose combination with plasma protein and passed into the liver, kidney and spleen, there was none in the pancreas. Taylor, Striven and Reid, in 1931,<sup>46</sup> induced hemochromatosis by depancreatizing a cat, and giving it a poorly balanced diet. This was the first recorded experimentally produced hemochromatosis. Polson<sup>38</sup> later extended his original experiment and gave iron subcutaneously and intraperitoneally for periods ranging from one to four years. Hemosiderin was deposited in the tissues, but no cirrhosis or hemochromatosis were produced. Menkin in 1934<sup>35</sup> gave intravenous injections of ferric chloride to rabbits for a number of weeks and produced hemosiderosis, showing that hemosiderin is not solely a product in the degradation of the hemoglobin but may result from a release of iron in the body fluids. In 1946, Herbut, Watson, and Perkins<sup>20</sup> showed that in 2 of their 30 rabbits with alloxan diabetes there was an associated cirrhosis of the liver, and to a lesser extent of the pancreas. Here, for the first time, two of the three important manifestations of hemochromatosis were noted, produced experimentally in animals by a single agent. The third manifestation, namely hemosiderosis, was produced by feeding reduced iron to the surviving diabetic rabbits.

### *Relationship of Hemochromatosis to Metabolic Diseases*

In 1945, Gillman, Mandelstam and Gillman<sup>15</sup> pointed out the relationship of hemochromatosis to nutritional disturbances in pellagrins. Their study was based upon histologic examination of 500 liver biopsies in South African natives. They emphasize in subsequent studies<sup>16</sup> that the excess of iron in the liver cell in cytosiderosis (hemochromatosis) can arise in only a limited number of ways, namely by the increased mobilization of iron from the red corpuscles and other bodily stores, by increased absorption and diminished excretion, and by abnormal utilization with normal absorption. Excessive destruction of the red blood cells has long been regarded as a potential cause of cytosiderosis of the liver. In pernicious anemia and the hemolytic anemias,<sup>45</sup> there is hemosiderosis but no cirrhosis. In malaria,

the liver and Kupffer cells are loaded with iron pigment but cirrhosis is absent.<sup>5</sup> In attempted experimental hemochromatosis, by the parenteral administration of colloidal iron to animals, the reticulo-endothelial system first becomes heavily laden with this metal, and only after a period of days or even months do the liver cells begin to accumulate iron. From this it may be concluded that the iron first accumulates in the Kupffer cells and only later does it appear to any extent in the liver cells. It has been suggested that cytosiderosis is in all probability due to a disturbed intracellular metabolism affecting the mitochondria, induced in some inexplicable way by a poor diet. Kremer<sup>28</sup> has shown an increase in the iron pigment in the liver of hibernating and starving frogs. Gore,<sup>17</sup> in 1939, reported the occurrence of pellagra and hemochromatosis. Taylor, Stiven and Reid<sup>46</sup> produced cytosiderosis in the cat by a badly balanced diet, and the Gillmans<sup>16</sup> have further focused attention on this relationship.

In reviewing 115 autopsied cases of cirrhosis of the liver, Herbut and Tamaki<sup>21</sup> found that 12 had diabetes mellitus. They brought out the fact that various combinations of cirrhosis of the liver, pigmentation, fibrosis of the pancreas, and diabetes mellitus do occur, and that the gradations from one to the other are so subtle that it is often difficult to know exactly when to apply the term hemochromatosis.

The deposits of hemofuscin are explained as part of a general disturbance of the metabolism of melanin, a pigment similar to hemofuscin. The hemosiderosis of the adrenals may in some way be related to this. The cause is probably ultimately referable to the abnormal metabolism in the parenchymatous cells and it is probably for this reason that sclerosis of some degree develops in so many of the organs and seems to vary in intensity with their levels of metabolism.

#### *Association of Hemochromatosis with Multiple Blood Transfusions*

The first case of hemochromatosis associated with multiple blood transfusions was reported by Kark in 1937.<sup>25</sup> This occurred in a 39 year old male who had received over 290 transfusions during a period of nine years. Bomford and Rhoads<sup>2</sup> reported 3 cases of a series of approximately 60 patients who had "refractory anemias." They were as follows: a 51 year old male who received 13 transfusions in one year, a 38 year old male who received 12 transfusions in sixteen months, and an 18 year old male who received 38 transfusions in five years. MacKey<sup>31</sup> reported a case of hemochromatosis in a 50 year old male who had received 39 transfusions in three and one-half years. Zeltmacher and Bevans<sup>48</sup> reported a 65 year old male who received 12.8 liters in one year. Humphreys and Southworth<sup>23</sup> reported a 58 year old female who had 52 transfusions in twenty-two months. Chesner<sup>8</sup> reported a 14 year old boy who had 12 transfusions over a period of nine months. The present report adds 5 cases: a 47 year old Negro female who had 75 transfusions in a period of four and one-half years, a 37 year old white female who had 65 transfusions in nine months, a 19 year old white female who had 21 transfusions in nine months, a 73 year old white female who had 23 transfusions in two years, and a 49 year old white female who had 58 transfusions in two years, bringing the total number of reported cases to 13.

## DISCUSSION

Hemochromatosis is a metabolic disease characterized primarily by an abnormally great retention and deposition of iron throughout the body. The iron is deposited in the form of hemosiderin. It has the greatest affinity for gland cells but nearly all tissues are ultimately affected. Two explanations, neither mutually exclusive, might explain the mechanics of the disturbed pigment metabolism according to Sheldon. Thus, "both pigments (hemosiderin and hemofuscin) might be the result of a disturbance of the normal changes in intracellular chemistry which are dependent on the process of ageing of the cell, or the hemosiderin might be due to an anomaly in the metabolism of the intracellular respiratory pigments whereby, though themselves of normal structure, the iron involved in their wear and tear is able to enter the cell without hindrance in either normal or increased quantity but becomes so altered while in the cell that it cannot be excreted, and consequently accumulates until the cell is distended to bursting point." Regardless of the pathogenesis of endogenous hemochromatosis, it is evident from animal experiments that the administration of iron parenterally results in its deposition in excess quantities, first in the reticulo-endothelial system and later in the parenchymal cells. Though this is sequentially the reverse of what supposedly happens in endogenous hemochromatosis the end results appear to be indistinguishable. Once extensive hemosiderosis occurs, the remainder of the picture develops as a consequence of the irritation of the foreign substance, and whereas the hemosiderosis in itself is apparently innocuous, the secondary fibrosis it produces is ultimately fatal. Fibrosis of the pancreas and the resultant diabetes with its complications account for about 50 per cent of the deaths while the fibrosis of the liver accounts for about 18 per cent (due to hematemeses, hepatic failure, and liver carcinoma).

It is not the purpose of this paper to evaluate the etiologic theories of endogenous hemochromatosis. Suffice it to say that the question relating to the ultimate cause of the iron retention has so far remained unanswered. It seems to us that the only *ultimate* cause can be *increased absorption of iron from the gastrointestinal tract*. Whether this absorption in turn is dependent upon an increased permeability of the cells of the gut, or an increased absorptiveness due to a low serum iron, as the work of Sachs, Levine and Griffith<sup>41</sup> would indicate, cannot be concluded from presently available evidence. Nor is it as yet clear, should this finding be substantiated, whether this is a primary defect or secondary to an increased 'avidity' of the parenchymal cells for iron. It is certain, however, that simple blood destruction cannot explain hemochromatosis either theoretically or on the basis of clinical or experimental evidence, since it is known that conditions characterized by increased blood destruction and hemosiderosis (such as hemolytic anemias, malaria, pernicious anemia, etc.) are not accompanied by fibrosis.

If we postulate, as indeed we must, that an increased amount of iron is present before hemochromatosis can occur, then, theoretically at least, any overloading of the organism with iron should result, at first in hemosiderosis, and ultimately hemochromatosis. The animal experiments referred to above bear out this hypothesis and probably failed to produce the complete picture because of insufficient insult and/or inadequate time.

Considering the rarity of hemochromatosis, the development of this disease in anemic patients who have had multiple transfusions appears to be more than simple coincidence. It is possible that the disturbed intracellular metabolism resulting from the anoxia due to the anemia may be contributory, making proper disposal of the iron pigment impossible. Thus, there would be an accumulation of hemosiderin, with eventual fibrosis added to the previously deranged cellular metabolism.

Theoretically, hemosiderosis should be producible in many ways and by any means which will increase the iron in the body over physiologic storage levels. This should be more readily accomplishable in the male whose iron excretion is much more limited than that of the female. Perhaps this is one of the explanations for the remarkable disparity between the incidence of hemochromatosis in the two sexes. The female has the opportunity, by pregnancies and menstruation, to lose quantities of blood which would tend to keep the accumulation of iron down to a point where, at least until the menopause, the hemosiderosis would be retarded. The age incidence of the disease, too, fits this concept, since to develop the full blown disease at the age of 50 (assuming the condition to be congenital) there would have to be an average daily excess positive iron balance of from only 1 to 2.5 milligrams.

Many more transfusions would have to be given, to administer enough iron to compare quantitatively with the amounts demonstrated in hemochromatosis, than the numbers either in our own cases or those previously reported, since a transfusion of 500 cc of blood contains approximately only 250 milligrams of iron. A satisfactory explanation for the recovery of 45 grams of iron in the liver in Chesner's case,<sup>8</sup> after having received a total of 6 liters of blood or 2.75 Gm of iron, on the basis of retention of transfused iron is still difficult to answer. We must, therefore, search for other factors which might be operative. Poor nutrition has been incriminated both clinically and experimentally, but we have no evidence of this in the present series. The only common denominator of our and other reported cases is anemia, regardless of type, for which the transfusions were given. As has been mentioned before, it is not inconceivable that the anoxia due to the anemia plays a contributing role by disturbing the intracellular metabolism, and thereby making the cell more susceptible to the deposition of iron. Significant additional amounts of iron are made available to these patients by oral administration which is universally done in the attempt to combat the anemia.

That the cases under discussion differ from the classic 'idiopathic' hemochromatosis is indisputable. It is, however, worthy of note that there are enough of the prime features in common to suggest that these cases represent intermediate or early forms which in time would probably show more and more of the classic features of the disease, since the typical case differs only in degree and extensiveness. Whereas classic hemochromatosis is characterized by fibrosis of the liver, pigmentation of the skin, diabetes mellitus, and sexual hypoplasia, of the cases reviewed here only 2 had clinically demonstrable diabetes and only 5 had skin changes worthy of note. There was no significant sexual hypoplasia in any of the cases. However, in 9 of the 13 cases there was a significant enlargement of the liver, and in all 12 of the 13 cases in which either autopsy or liver biopsy were obtained a

TABLE 1—Cases of Exogenous Hemochromatosis

Author and Date	No of case	Sex and Age	Primary Diagnosis	Duration	Number of Transfusions	Initial Blood Count	Last Blood Count	Marrow Findings	Skin	Liver	Spleen	Diabetes
R M Kark 1937	1	39 male	Aplastic Anemia	9 years	290 (plus)	Hgb 50% RBC 2 50 WBC 3,500	Hgb 50 to 70%	Many red cells present but normal hematopoietic tissue somewhat deficient Eosinophils and round cells plentiful Extreme replacement of marrow with fat	Peculiar slate gray color Pigmentation of conjunctivae and teeth	Enlarged down to umbilicus	Not enlarged	Fasting blood sugar 150 mgs /100 c cm
R R Bonford and C P Rhoads 1941	2	51 male	Aplastic Anemia	16 months	15	Hgb 30% RBC 1 40 WBC 1,150	RBC 1 00	Slightly hypocellular with isolated areas of active hemopoiesis No megakaryocytes	Blackish brown pigmentation of hands and arms	1,500 Gms	Not enlarged	
	3	68 male	Pseudo Aplastic Anemia		12	RBC 1 50	RBC varied from 1 0 to 2 00 WBC 2,000 to 5,000	Hypercellular Reduced number of megakaryocytes	Severe jaundice Brown pigmentation of skin	1 840 Gms	130 Gms	
	4	21 male	No Diagnosis	8 years	55	Hgb 26% RBC 1 30 WBC 3,850	Hgb 30% RBC 1 40	A partly mature marrow with few megakaryocytes	Brown pigmentation	Not enlarged	Slightly enlarged	Present
R Mackay 1942	5	46 male	Aplastic Anemia	34 years	39 8 liters	Hgb 22% RBC 1 05 WBC 4,100	RBC varied between 1 10 to 2 70	Increased in amount Deep brown color Increased granules in all stages Increased lymphocytes Normal megakaryocytes Only occasional nucleated red blood cell		2,400 Gms	450 Gms	

K /elt marker and M Bevans 1915	6	65 male	Pseudo Aplastic Anemia	1 year	12.8 liters	Hgb 36% RBC 1.40 WBC 4,900	Varied from RBC 1.15 to 2.50 Hgb 34% to 54% WBC 2,600 to 6,100	Very cellular with RBC WBC ratio 1:1 Marked shift to left in nucleated RBC and dimin- ished numbers of mature granulo- cytes	Gray-brown	3,050 Gms	250 Gms	Tasting blood sugar 110 and 130
G. H Hum phreys and H South worth 1915	7	58 female	Aplastic Anemia	3 years	52	Hgb 10% RBC 1.00 WBC 10,000	Hgb 95% RBC 4.00	Mild hypoplasia of marrow but no ab- normal cells	Normal	6 cm below costal mar- gin	Not palpable	None
C Chesner 1916	8	11 male	"Banti's Dis- ease"	7 1/2 months	6 liters	Hgb 3.9 Gm RBC 2.50 WBC 4,000	Hgb 6 Gm RBC 2.50 WBC 3,000	Hyperplasia of the erythroid ele- ments	Pale	2,350 Gms	3 fingers down from costal margin	
S O Schwartz and S A Blumen- thal 1917	9	19 female M P	Congenital hypoplasia of kidneys with chronic glomerulo- nephritis	9 months	21	Hgb 44% RBC 2.24 WBC 6,500	Hgb 50% RBC 3.01 WBC 6,000	Relatively acellular, made up mostly of granulocytic cells with right shift	Scaly maculo- papular eruption	5 cm below the costal margin 1,665 Gms	Not enlarged 165 Gms	None
	10	73 female R H	Aplastic Anemia	2 years	23	Hgb 50% RBC 1.72 WBC 9,000	Hgb 19% RBC 1.12 WBC 6,900	Hypoplastic mar- row	Pale	900 Gms	50 Gms	None
	11	49 female C K	Anemia with hepato- spleno- megaly	2 years	58	Hgb 36% RBC 2.50	Hgb 9% RBC 1.30 WBC 14,100	Hyperplastic mar- row with moderate replacement by large primitive cells which may represent eryth- roid elements	Dark color	1,980 Gms	520 Gms	Present



TABLE 1.—*Continued*

Author and Date	No. of case	Sex and Age	Primary Diagnosis	Duration	Number of Transfusions	Initial Blood Count	Last Blood Count	Marrow Findings	Skin	Liver	Spleen	Diabetes
	12	37 female J B	Aplastic Anemia	9 months	65	Hgb 44% RBC 2,34	Hgb 22% RBC 1.09	Hypocellular marrow with small islands of normal erythropoiesis	Pale	Not enlarged	Not palpable	None
	13	47 Negro female V G	Anemia with hepato spleno megaly	4½ years	75	Hgb 12% RBC 0.70 WBC 9,300	Hgb 60% RBC 3.76 WBC 25,600	Hypocellular marrow Megakaryocytes normal Remarkable increase in primitive red cells No megaloblasts noted Shift in granulopoiesis, with numerous giant band forms and metamyelocytes Increased number of eosinophils	Dark	8 cm below the costal margin 3,160 Gms	4 cm below the costal margin 780 Gms	None

CASE 1 Autopsy Autopsy refused Skin biopsy Brown, iron containing pigment in secretory cells of the sweat glands, lesser amount in the sebaceous glands and diffuse distribution in the connective tissue and basement membrane Remarks Patient began to develop the peculiar skin changes of hemochromatosis and enlarged liver about 8 years after the beginning of the transfusion series

CASE 2 Autopsy Hemosiderosis and fibrosis of liver, pancreas and lymph nodes Immature hypoplastic marrow Liver firm, golden brown cut surface, pancreas deep brown in color Considerable hemosiderosis and multilobar fibrosis of liver and pancreas Extraductal hemopoiesis in spleen Remarks Prior to transfusions patient was treated unsuccessfully with iron, liver copper and vitamins

CASE 3 Autopsy Broncho pneumonia of right lower lobe Spleen brownish purple cut surface, liver abundant golden brown pigment around portal areas Pancreas deep brown in color Considerable hemosiderosis and multilobar fibrosis of liver and pancreas Extraductal hemopoiesis in spleen Remarks History of severe nosebleeds from age of 5 to 7, bleeding tendencies and purpura with splenectomy at age of 13

CASE 4 Autopsy Typical findings of hemochromatosis with hemosiderosis and fibrosis of liver and pancreas Remarks Periportal fibrosis and free iron demonstrated in reticulo-endothelial cells and parenchymal cells Evidence of hemochromatosis noted in pancreas lymph nodes and heart Remarks Previous history was negative

CASE 5 Autopsy Liver orange brown loaded with hemosiderosis Spleen had a small amount of iron pigment Pancreas was heavily pigmented and fibrotic Prostate, lymph nodes, adrenal thyroid and heart contained pigment Marrow showed evidence of regression toward a more immature state Lungs emphysematous Right heart enlargement

CASE 6 Autopsy Iron staining pigment in liver pancreas bone marrow thyroid adrenals spleen and lymph nodes Remarks This patient had a profound depression of erythrocyte formation for many years and had to be maintained on blood transfusions following removal of a mediastinal tumor but red count went back to normal and she remained well for one year when she developed an abscess in her thigh followed by acute necrotic jaundice and death



hemosiderosis and fibrosis of the liver were found. Of equal importance was the finding of both hemosiderosis and fibrosis in all 11 cases where pancreatic tissue was examined. In the thirteenth case, only a skin biopsy was obtained.

It seems important to recognize that in dealing with simple hemosiderosis, exogenous hemochromatosis with secondary fibrosis, and hemochromatosis of the idiopathic type we are dealing with varying gradations of the same aberration. From observations made in our own cases, together with the findings of previous investigators (both clinical and experimental), it may be concluded that the deposition of excess amounts of iron will lead to irritation, fibrosis and ultimately to destruction. Whether this is precisely the sequence of events that occurs in hemochromatosis is unknown and seems to be somewhat controversial. It is not unlikely that other factors besides the simple deposition of iron may be responsible for the fibrosis. We are, of course, dealing here with a highly selected group of cases, all of whom had a profound anemia which undoubtedly damaged the liver parenchyma as well as the parenchyma of other organs, especially those with high metabolic needs. It is important in this connection to re-emphasize the fact that the organs most involved in hemochromatosis are those with high metabolic levels and oxygen consumption. The factor of hemolytic transfusion reactions, not by virtue of hemolysis but because of the accompanying fever and further parenchymatous tissue damage, may well have been an aggravating circumstance. Although this was not of universal occurrence its importance cannot be minimized.

One of the striking differences between the cases here reviewed and those of typical hemochromatosis is the marked difference in the ratio of males to females. In endogenous hemochromatosis, the ratio is about 20 to 1 in favor of males. In the present series, there are 7 males and 6 females, while in our own series all 5 were females. Of further interest is the fact that, whereas the classic picture is practically unknown before the age of 20, there were 3 cases in the present group between 14 and 21 years of age.

Fully developed hemochromatosis, showing all the manifestations of the disease, will undoubtedly be encountered in time because of the increasing utilization of blood transfusions and the greater longevity of patients having hematologic dyscrasias. There seems to be a definite relationship between the degree of hemosiderosis and fibrosis and the factors of time and number of transfusions. By and large, the patients receiving only relatively few transfusions or transfusions over a relatively short period of time seem to show the least involvement, whereas patients who receive many transfusions over long periods of time (cases 1, 4, 5, 11 and 13, table 1) will have the greatest amount of involvement. It is obvious from the foregoing discussion that patients to whom blood is given for the purpose of replacing lost blood will have no tendency to develop hemochromatosis since the iron is simply being replaced as it is lost from the body.

Our own interpretation of endogenous hemochromatosis is that it is a metabolic disease in which excess amounts of iron are absorbed from the gastrointestinal tract. Whether this excess iron is absorbed due to the fact that there is a breakdown of the barrier in the gut or whether there is an abnormally low serum iron, either as a distinct abnormality or due to the avidity of various tissues for iron, we are

is yet unprepared to say. In those cases where iron is introduced into the body parenterally either in a free or combined state (as in the form of red cells which are eventually broken down to hemoglobin) and becomes stored, it is gradually laid down in the various storage depots because no mechanism of excretion exists. When the storage depots become overfilled the parenchymatous cells, which under normal circumstances do not contain iron, will take up the excess. We believe that this same sequence of events occurs in endogenous hemochromatosis and hence the greater incidence in males and the reason for the occurrence of the disease in later life.

It is worthy of note that the underlying disease for which the transfusions are given is not of prime importance. Diagnoses included true aplastic anemia, pseudo-aplastic anemia, chronic uremia, and others. In this connection it is particularly noteworthy that there is never a significant anemia in the classic hemochromatosis. Therefore, the assumption that these cases had hemochromatosis and were complicated by anemia, for which in turn the transfusions were given, is fallacious.

The therapeutic suggestion of venesections and iron deficient diet presents itself in the management of hemochromatosis. It is not thought that this regime will be curative, but may serve, by reducing the hemosiderin deposits, as an ameliorating factor in the course of the disease.

### CASE REPORTS

#### *Case 1 Marilyn P.*

Patient was a 19 year old white female who entered the hospital with a history of weakness, dyspnea, headaches, anorexia, sore throat and sore tongue, generalized pruritic scaly dermatitis, and recurrent bouts of epistaxis for a year. Past history revealed that she had scarlet fever during childhood and frequent sore throats subsequently. During the year preceding admission she had been treated with iron for anemia. On examination she was found to be poorly developed, but well nourished. The skin was covered by a pale, tan, finely scaly, maculopapular eruption involving the trunk and extremities. Blood pressure was 120/60. The temperature was normal. Transitory attacks of twitching and convulsions with mental aberrations were noted. Significant laboratory findings on admission were Hgb, 44 per cent, RBC, 2.24, WBC, 6,500, polys, 82 (bands 1), eosinophils, 1, lymphocytes, 10, monocytes, 7. The red cells showed slight anisocytosis and poikilocytosis, the granulocytes, slight toxicity. Urinalysis albuminuria 2 plus, with 15-20 WBC and occasional RBC per HPF. NPN, 304 mg per 100 cc, creatinine, 16.7 mg per 100 cc, total proteins, 7.2 Gm per 100 cc, serum calcium, 5.6 mg per 100 cc, inorganic phosphorus, 11.2 mg per 100 cc. Marrow was relatively acellular and consisted mostly of granulocytes which showed a right shift. She was given Blaud's pills, multiple vitamins, intravenous fluids, and sodium and calcium therapy.

During the next several months she was in the hospital on three other occasions. During these, 18 transfusions of 500 cc blood were given. The final admission was eight and one half months after the first because of an exacerbation of the uremic symptoms of weakness, nausea, anorexia, skin rash and epistaxes. The blood pressure had risen to 172/126. There were rales at both lung bases, an ascites, a palpable, tender liver 4 cm below the costal margin, and ankle edema. The urine now had 4 plus albumin. Hgb, 50 per cent, RBC, 3.01, WBC, 6,000, polys, 83 (bands 13), eosinophils, 1, lymphocytes, 12, monocytes, 4. NPN, 161 mg per 100 cc, creatinine, 14.8 mg per 100 cc, calcium, 7.5 mg per 100 cc. She gradually became more stuporous and died in uremic coma nine months after first entering the hospital. Three additional transfusions of blood were given during this admission.

On postmortem examination, bilateral hypoplastic kidneys were found. These showed a chronic recurrent glomerulonephritis which was chiefly intracapillary. There was also hypoplasia of the renal arteries and the aorta. In the liver (1,665 Gm), there was marked hemosiderosis with reactive periportal

fibrosis and bile duct proliferation. The spleen (165 Gm) showed a chronic reactive hyperplasia with hemosiderosis. There were also hemosiderosis of the lungs and lymph nodes and a slight interstitial

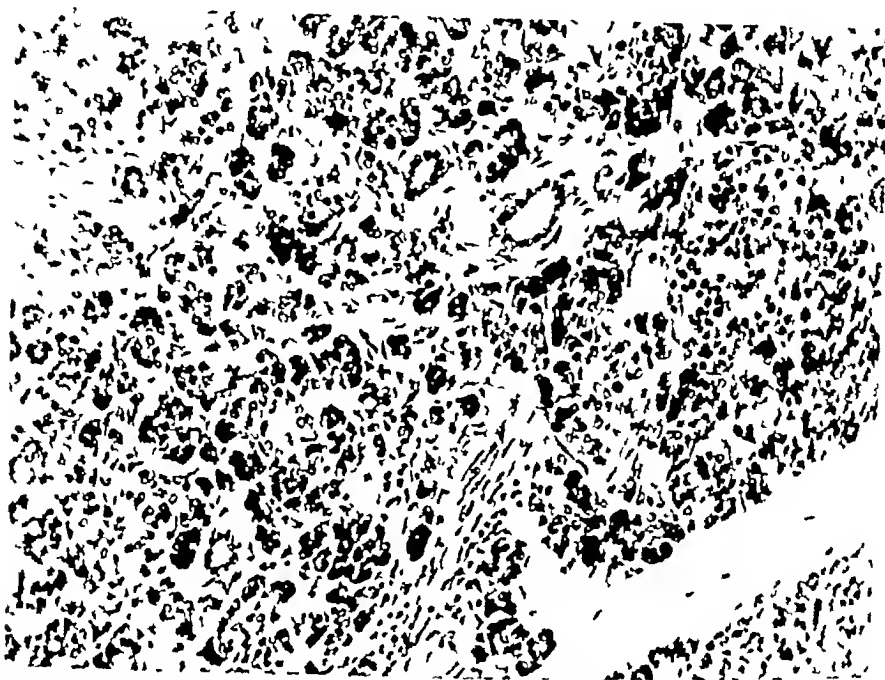


FIG. 1. Slight interstitial pancreatitis and increase in fibrous tissue (H and E Stain) Case 1 160 X

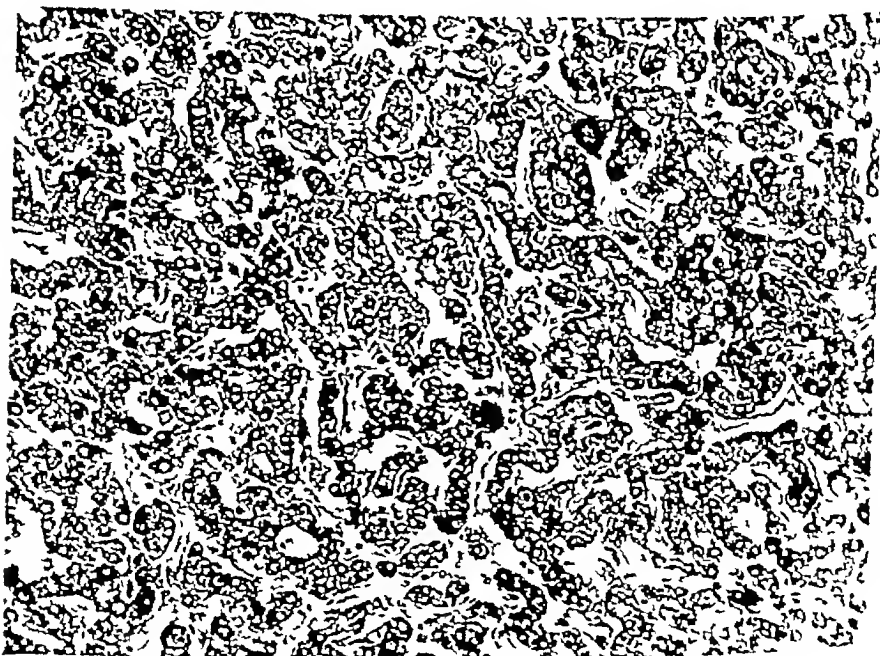


FIG. 2. Liver, showing slight increase in connective tissue (Mallory stain) Case 1 160 X

pancreatitis. Other findings were the marked hypertrophy and dilatation of the heart, bilateral hydrothorax, ascites, anasarca, uremic pericarditis, beginning uremic colitis, and hemorrhagic infarct of the lower lobe of the right lung.

*Comment* This 19 year old girl had a severe anemia secondary to chronic uremia. During the nine month observation period, she received 21 transfusions of 500 cc blood in addition to ferrotherapy. Her liver, lungs, lymph nodes and spleen showed hemosiderosis. The liver, however, already had some periportal fibrosis at the time of death. It is difficult to say whether the pancreatitis was part of the picture. There were no skin changes or diabetes in this case. It is of great interest that periportal fibrosis should be demonstrable as early as nine months after the start of transfusions. There is nothing in the underlying disease which in itself should have predisposed this patient to liver damage or fibrosis other than the anemia and the hemosiderosis.

#### *Case - Ret a II*

Patient was a 73 year old white female whose main complaints were weakness for five months, fatigue, and pins and needles sensations in the fingertips for four weeks. Some dyspnea on exertion, coldness of the feet, belching after meals, a dry tongue, and an 11 pound weight loss had also been noted. Past history was noncontributory. On examination she was found to be well developed and well nourished, pale, and with a yellowish tint of the skin. The tongue was coated. There was a loud, rough, systolic murmur heard at the apex, which was transmitted to the neck and axilla. The blood pressure was 135/70. There was some tenderness in the left upper quadrant. Liver was felt 1 centimeter below the costal margin. Large varicosities were noted in the lower extremities. There was an ankylosis of the left hand on an orthopedic basis. Significant laboratory data were: negative urines, stools and serologic findings. Gastric analysis after subcutaneous histamine: free acid, 29°, total acid, 50°. Normal BMR and EKG. NPN, 29 mg per 100 cc, blood sugar, 77 mg per 100 cc, cholesterol, 118 mg per 100 cc, cholesterol esters, 70 per cent. X rays: Gallbladder showed good dye concentration and the roentgenologic examination of the gastrointestinal tract was negative. Blood count on admission was as follows: Hgb, 50 per cent, RBC, 1,72, WBC, 9,000, polys, 56, eosinophils, 2, lymphs, 34, monocytes, 8, anisocytosis, 2 plus, microcytosis, 4 plus. Platelets, 110,000, fragility test, normal. Marrow: hypocellular, with marked diminution of both erythropoiesis and granulopoiesis and increase in lymphoid elements. She received liver extract and one blood transfusion without benefit during her four week hospital stay.

She was rehospitalized nine months later and on this admission it was learned that she had been taking phenobarbital every night for the preceding eight months, and on and off for the previous six years. Her symptoms were the same as before but her arthritic pains had increased and a rash had appeared over the back and shoulders. She was again extensively studied, received two transfusions and was sent home after four weeks. There were four other admissions, the last one twenty-three months after the first. This admission was because of a pneumonia and pyelonephritis. Five blood transfusions were given during this admission, but despite these and other supportive measures she progressively failed and died two and one half months later. She had received a total of 23 blood transfusions during the two years of observation.

On postmortem examination the following pertinent findings were noted: hypoplasia of bone marrow, hemochromatosis of liver, spleen and pancreas, icterus with purpura, left pyelonephritis with cortical abscesses, moderate coronary artery arteriosclerosis with myocardial fibrosis, healed endocarditis of tricuspid, mitral and aortic valves, old pleuritic adhesions (rt) with *Durchwandrung* perihaptic adhesions.

*Comment* This 73 year old patient was studied over a two year period, during which time she received 23 blood transfusions. Many of the transfusions were followed by severe febrile and hemolytic reactions, which, in retrospect, were probably due to the then unknown Rh factor. What aggravating role these reactions played it is impossible to say, but they may have produced enough liver damage to have at least contributed to the ultimate fibrosis of the liver and other organs. It is

not thought significant that the blood was rapidly destroyed during these reactions rather than in the normal way. There is no evidence to indicate that the underlying

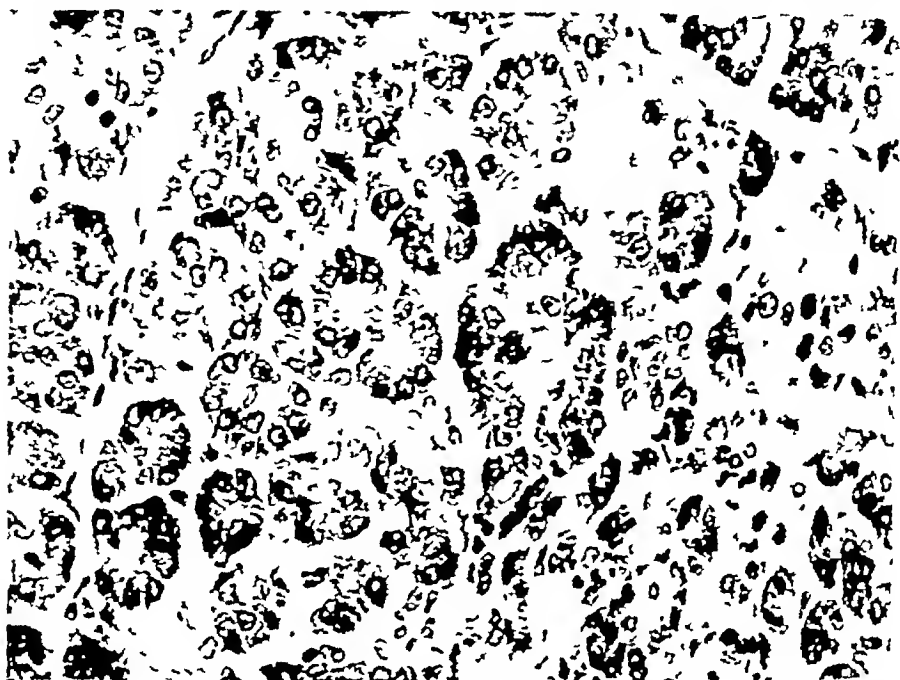


FIG 3 PANCREATIC FIBROSIS (H AND E STAIN) CASE 2 320 X

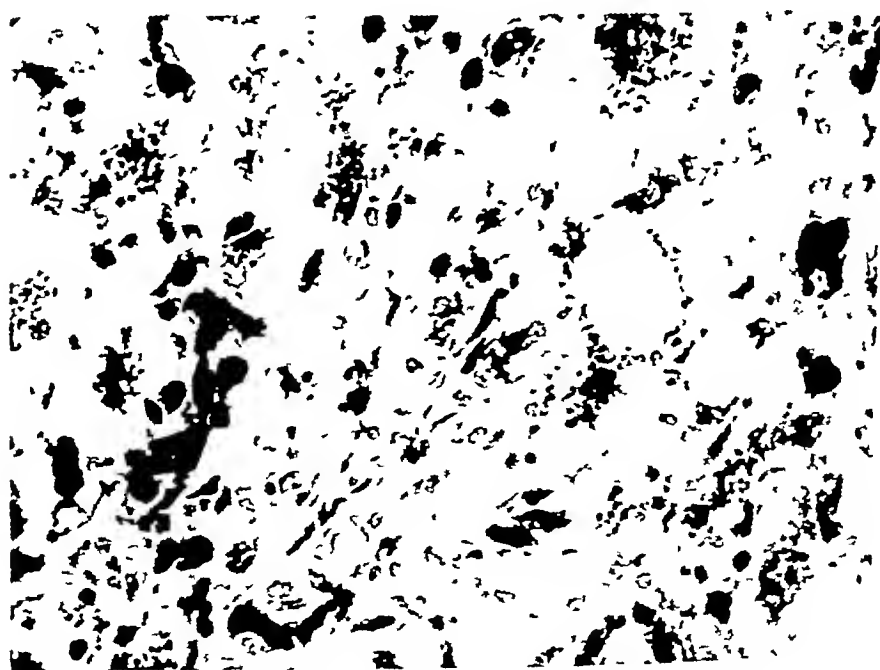


FIG 4 Increased pigment in liver cells (Basic fuchsin and nitric acid stain) Case 2. 320 X

aplastic anemia was in any direct way related to the hemochromatosis. This patient had no clinical evidence of diabetes or skin involvement.

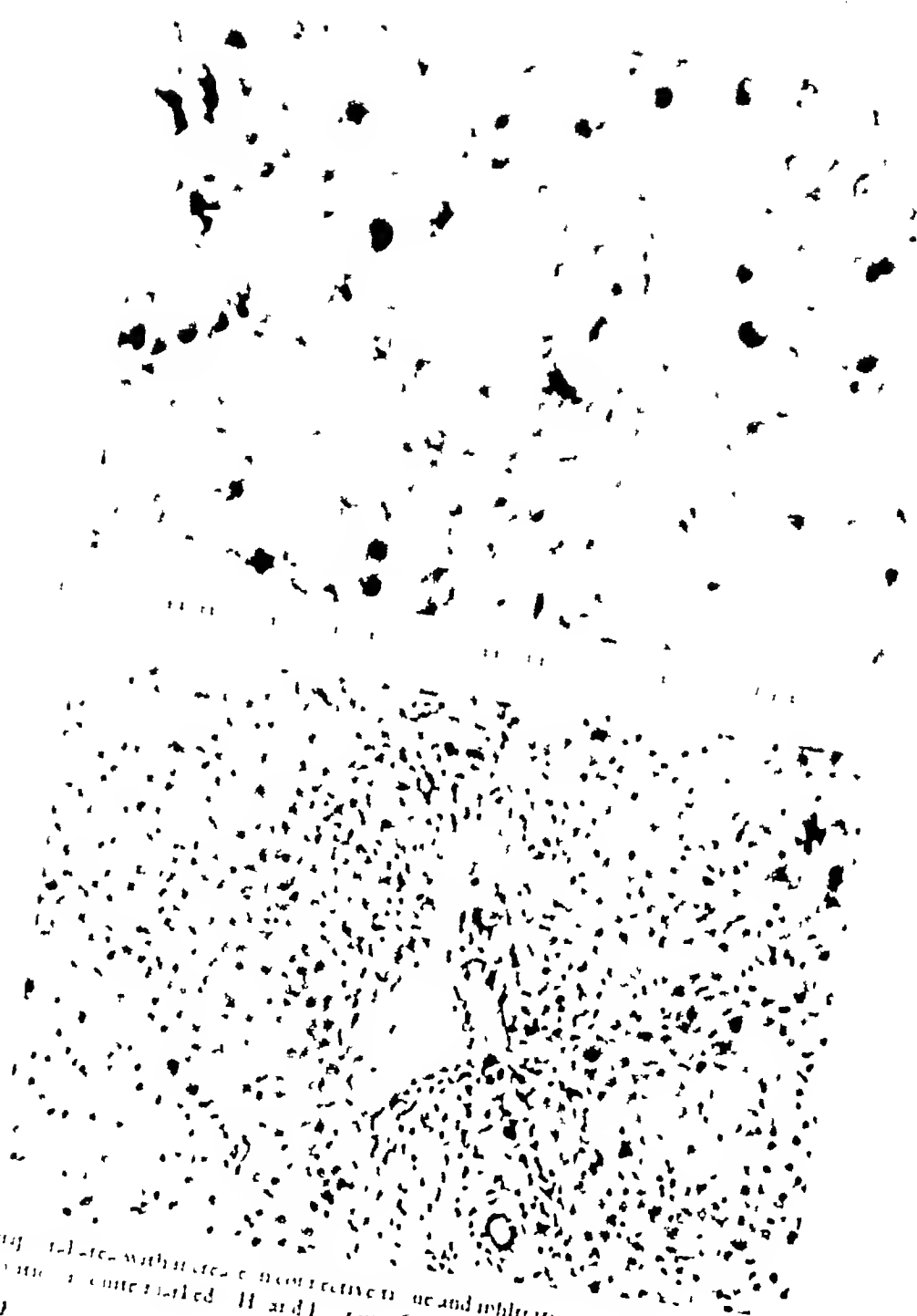


Figure 1. Pericardial tissue with extensive non-specific inflammation and infiltration with round cells. Hemosiderin pigment deposits are also noted. (H and E stain, Case 363)

Patient was a 49-year-old white female who entered the hospital with a history of weakness, palpitation, dyspnea, nervousness, anorexia and a 30 pound weight loss in the previous two years. During the



four months before her present hospital admission she had received 11 transfusions for anemia at a private hospital. Except for a right sided oophorectomy in 1917, her previous history and family history were noncontributory. She took milk of magnesia quite frequently and occasionally aspirin for headaches. She was found to be well developed and well nourished. The skin was pale and had a slightly icteric hue. There were a few small discrete submaxillary lymph nodes, a marble sized node in the left axilla, and a few palpable nodes in the inguinal region. The heart was enlarged to the left. A soft systolic murmur was heard at the apex and there was a systolic murmur at the base. The liver was moderately hard and could be felt 3 cm. below the costal margin. The spleen was felt 2 cm. below the costal margin.

Laboratory findings of interest were the following: Hgb, 36 per cent (5.6 Gm), RBC, 2,500,000, WBC, 7,900, polys, 59, lymphs, 27, monocytes, 13, eosinophils, 1. Platelets, 170,000. Urines, blood serology and stools negative. Serum protein, 7.4 Gm, inorganic phosphorus, 4.7 mg, acid phosphatase, 0. Icterus index, 9. EKG slightly abnormal, with left axis deviation. Sternal marrow revealed an extraordinary cellularity, the megakaryocytes were normal, granulopoiesis was intact, erythropoiesis was accelerated to a remarkable degree and showed a marked left shift with very primitive erythropoiesis predominating. X-rays of the bones and the gastrointestinal tract were negative. The chest x-ray showed a boot shaped heart and accentuated hilar markings bilaterally, but otherwise lung fields were normal.

She was given 6 blood transfusions during a period of eighteen days, and was discharged, significantly improved both clinically and hematologically. No satisfactory diagnosis was established.

She was readmitted six months later because of a recurrence of weakness. There were no significant changes in her physical findings. The blood findings now were: Hgb, 36 per cent (5.6 Gm), RBC, 2,710,000, WBC, 10,400, polys, 49 (bands 37), myelocytes, 2, metamyelocytes, 10, eosinophils, 1, basophils, 1, irritation cell, 1, lymphocytes, 18, monocytes 18. This time she received 8 blood transfusions during a three week period.

Two months later she was admitted to another hospital. Her liver now was 9 cm. and the spleen 6 cm. below the costal margin. Hgb, 23 per cent (3.6 Gm), RBC, 1,400,000, WBC, 6,300, polys, 63 (bands 45), lymphs, 9, monocytes, 6, basophils, 1, myelocytes, 10, metamyelocytes, 11. BMR was +40. Otherwise, nothing new was found. Despite a total of 20 blood transfusions during this four month hospitalization her blood count still remained quite low.

Thirteen months after her first hospitalization she entered still another hospital. Here she received 13 more blood transfusions and splenectomy was performed. Her postoperative course was stormy, she developed pleural effusions and an hemopericardium, and died about six weeks later. Most significant single contribution clinically during this hospitalization was the discovery of a mild diabetes.

Postmortem examination revealed a pigment cirrhosis of the liver, hemosiderosis of the liver, stomach, adrenal glands, and lymph nodes. There was an interstitial fibrosis of the pancreas with extraordinarily large amounts of hemosiderin deposits involving especially the islands of Langerhans. The marrow was hyperplastic and the extirpated spleen showed myeloid metaplasia. Other findings were a fatty degeneration of heart and kidneys, subacute fibrinous pericarditis, chronic abscess of left upper quadrant of the abdomen, left pleural effusion, and septicemia (*staphylococcus aureus*).

*Comment* This 49 year old woman was studied at four institutions during her two years of illness. Every conceivable diagnosis was made and all types of therapies used in hematology were tried without success. Even postmortem examination failed to explain the cause of her anemia, or cast light upon the type of maturation failure seen in the primitive erythrocytes.<sup>42</sup>

During the two years of study she received 58 transfusions of blood, most of them uneventfully. Of greatest interest in this patient was the recognition of early diabetes some time before her death, explained pathologically by the large amounts of hemosiderin deposits in the islands of Langerhans and by the fibrosis in the pancreas. She had extensive hemosiderin deposition in most organs and, significantly, cirrhosis of the liver. This patient's skin was quite dark, having become more so during her last few months. Unfortunately no skin biopsy was obtained—one of

the disadvantages of retrospective investigation. It may also be said in retrospect that the splenectomy was not justified since the enlargement of the spleen was apparently secondary (due to compensatory extramedullary hemopoiesis) rather than the cause of the anemia. (This case has been previously published<sup>30</sup>)

#### Case 4 Jean B

Patient was a 37 year old white female who entered the hospital because of malaise, fatigability, dyspnea, ankle edema and palpitation for one month. She was actively bleeding vaginally on admission and had just been discharged from another hospital where one blood transfusion was given. For twenty-three years she had had menorrhagia lasting from one to four weeks, associated with dysmenorrhea for which she frequently took Anacin and Midol. There had also been many nose bleeds during the

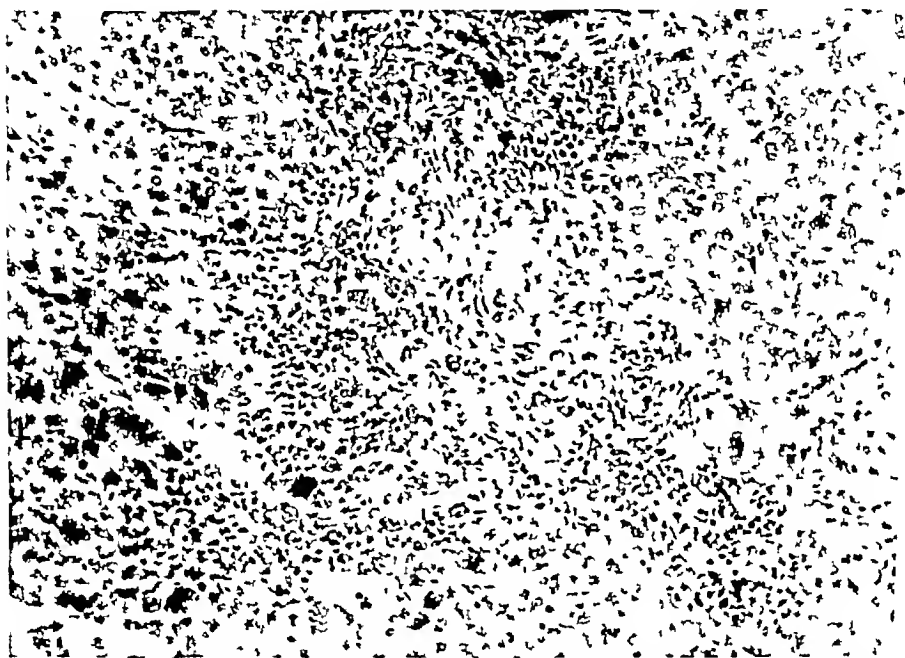


FIG 7 Periportal area showing increased hemosiderin pigment deposition (H and E stain) case 4 160 X

last several months. The patient worked in a pie factory most of her adult life. She had had an appendectomy and had never been pregnant, denied having had syphilis or having received anti-tuberc therapy. She was well developed and well nourished, and appeared pale and weak. The hair was dried. The pupils were equal but asymmetrical and did not react to light or accommodation. There was a loud, blowing systolic murmur heard at the apex and base of the heart. Neither the liver nor the spleen were palpable. The extremities were covered by numerous large ecchymotic areas. A lemon sized cystic mass was palpated in the left adnexa, and the uterus was in the cul-de-sac.

Only laboratory findings of significance were the negative serologic tests for syphilis, the hematologic findings, and the marrows. The blood revealed Hgb, 44 per cent (6.8 Gm), RBC 2.34, WBC 2,150, polys, 65 (bands 25), lymphocytes, 27, monocytes, 8. Platelets, 7,000 (two days after blood transfusion). Sternal marrow was replaced by fibrous tissue.

During the first three weeks in the hospital, 1,300 r units of Roentgen radiation were given to the area of the pelvis following which the uterine bleeding stopped. She also received 8 blood transfusions which elevated her count to Hgb, 67 per cent (10.4 Gm), RBC, 4.62, WBC, 3,050. For the next several months her condition was fairly good except for an occasional transfusion reaction. In five months she had received 44 transfusions, in spite of which her count had been gradually dropping, being Hgb 20 per cent, RBC 0.93, WBC 900 at this time. From this point her condition slowly deteriorated. Transfusion reac-

tions became quite frequent, even with washed red cells, epistaxes became troublesome and required packing, petechiae and ecchymoses became more numerous

Eight and a half months after admission, following her sixty-fifth transfusion, she developed a thrombophlebitis of the left arm. In spite of the usual methods of therapy, the process continued to spread and the patient expired eight days later. Permission to perform an autopsy was refused. Post mortem liver biopsy revealed a hemosiderosis associated with increased fibrosis of the periportal tissues (very early cirrhosis).

*Comment* This 37 year old woman had an aplastic anemia of unknown etiology. She had taken "Anacin" and "Midol" intermittently for years but none of the usually incriminated drugs or agents had played an etiologic role. We speculated on the possibility that this case might have represented one of those rare instances, if indeed such conditions occur at all, in which true marrow 'exhaustion' occurs from too protracted and severe a drain. Her menorrhagia dated back to the menarche 23 years previously. This is, of course, purely speculative, since we know nothing about the nature of the pelvic tumor or the blood findings at any time during the previous twenty-three years. Unfortunately, no postmortem examination was performed, but the liver biopsy showed unmistakable signs of early cirrhosis about nine months after the first transfusion. Actually, it cannot be assumed that this patient started depositing iron in the beginning since she was still bleeding and her transfusions served as replacement therapy. Thus, her early cirrhosis occurred in a period of less than eight months.

#### *Case 5 Victoria G*

The patient, a Negro female, was 43 years old when she first entered the hospital because of weakness, dyspnea, orthopnea and bronchitis of one year's duration. For six weeks before admission she had noted vague chest pains, moderate epistaxes, blood streaked sputum, and paresthesias of the hands and feet. She had had typhoid-malaria during childhood, and anti-luetic therapy with arsenic and bismuth some years previously. She was well developed and well nourished, quite pale, moderately dyspneic, and coughed frequently. The heart was enlarged both to the right and left, loud systolic and presystolic murmurs were heard at the apex and the systolic murmur at the base was heard transmitted to the vessels of the neck. There was dullness at both bases, breath sounds were distant and rales were noted in the right base. The liver was felt 4 cm. below the costal margin and had a sharp tender border. The spleen was not palpable. External hemorrhoids were present. Moderate nonpitting edema of both ankles was noted. Fundoscopic examination revealed nothing remarkable.

Examination of urines, stools and serologic blood tests were noncontributory. The gastric contents contained 40° free and 60° total acid. NPN, 26 mg. per 100 cc., creatinine, 1.5 mg. per 100 cc., icterus index 7 units. Hgb., 12 per cent (1.8 Gm.), RBC, 0.70, WBC, 9,300, polys, 71 (bands 15), eosinophils, 6, basophils, 2, lymphocytes, 21, hyperchromia 2 plus, anisocytosis, 3 plus. Tests for sickling were negative. The marrow was hypercellular, megakaryocytes were normal, primitive red cells dominated erythropoiesis, but there were no megaloblasts, granulopoiesis showed a slight left shift with numerous giant band forms and megalometamyelocytes, eosinophils were extremely numerous. X-rays of the chest confirmed the cardiac enlargement, while those of the gastrointestinal tract were negative. The patient was given digitalis, diuretin, ammonium chloride, parenteral liver extract and 3 transfusions of 500 cc. of blood. She felt better and left the hospital in three weeks. No diagnosis was established.

During the next four and one quarter years she was treated at three different hospitals, receiving a total of 50 blood transfusions and a course of bismuth and arsenic. At the end of this time she was readmitted to the Cook County Hospital with symptoms essentially similar to the original ones. She appeared older than her chronologic age of 47 and was quite pale and dyspneic. The liver now was 10 cm. below the costal margin, and was firm and tender. The spleen was palpable on deep inspiration. Mild pitting edema of the ankles was present. At this time she had Hgb., 15 per cent (2.3 Gm.), RBC, 0.69,

WBC 11,450, polys 68, bands 12, count 4000, hils 1, lymphocytes 27, monocytes 4. Urinalysis was negative. Icterus index 14 units. Serum proteins 6.5 Gm, albumin 3.2 Gm, globulin 3.3 Gm, blood

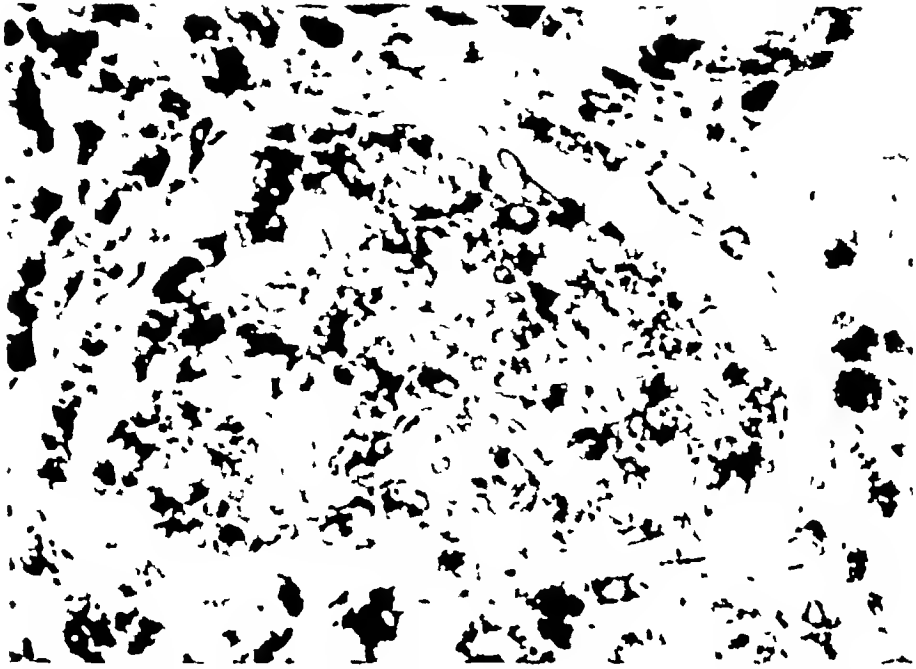


FIG. 8. Great increase in iron deposition both in pancreatic acini and islets of Langerhans (Iron Stain) Case 5 320 X

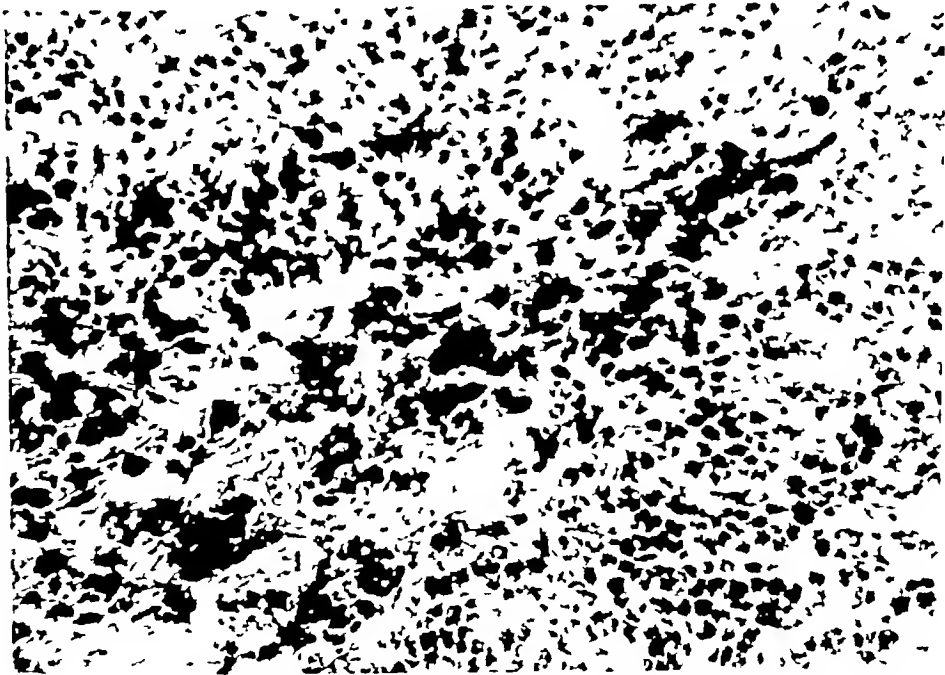


FIG. 9. GREAT INCREASE IN HEMOSIDERIN IN LIVER (IRON STAIN) CASE 5 160 X

cultures and blood and spinal fluid serology negative. In spite of 9 blood transfusions during an eighteen day period, supplemented by oral iron and parenteral liver therapy, her blood count remained at Hgb,

19 per cent, RBC, 0.93, WBC, 14,500, polys, 64 (bands 29), eosinophils, 13, lymphocytes, 21, monocytes, 1, metamyelocytes, 1. Marrow findings were unchanged.

At this time a splenic inhibition was considered as responsible for preventing the maturation of the erythrocytes and it was decided to perform a splenectomy. This was performed after a preliminary series of transfusions, elevating the blood count to Hgb, 58 per cent, RBC 3.68, WBC 6,300. In spite of the preparation and the administration of 1,000 cc. of blood during operation, the patient went into shock immediately after the removal of the spleen. She responded to antishock management and for the first few days postoperatively seemed to be in fair condition. However, she developed bronchopneumonia and died seven days after the operation. During this period, 3 more transfusions had been given. Her last blood count showed Hgb, 60 per cent, RBC, 3.76, WBC, 25,600, polys, 83 (bands 30), basophils, 1, lymphocytes, 15, monocytes, 1.

Pertinent postmortem findings were Hemosiderosis and cirrhosis of the liver (3,160 Gm), hemosiderosis of the pancreas and abdominal lymph nodes, hyperplasia of the marrow, multiple accessory spleens, terminal bronchopneumonia, fatty degeneration of the myocardium, and acute edema of the lungs. The extirpated spleen weighed 780 Gm and showed a marked diffuse fibrosis. About thirty accessory spleens, measuring from 2 mm to 4 cm, and weighing a total of about 80 Gm, were also removed at operation.

*Comment.* This 43 year old Negress had a profound anemia whose etiology, as that of case 3 which it most closely resembled, was never satisfactorily established.<sup>42</sup> The anemia was characterized by a hypercellular erythroblastic marrow which showed evidences of a maturation arrest at a primitive stage and did not respond to any of the known anti-anemia preparations. Life was maintained by substitution therapy consisting of 75 blood transfusions during four and one-half years. This patient did not develop diabetes. Whether skin changes occurred is not known. The remarkable enlargement of the liver was on the basis of the marked hemosiderosis and fibrosis. Undoubtedly these changes made her a worse surgical risk and contributed to the fatal outcome of the splenectomy.

#### SUMMARY

1. Five patients who died as a result of a variety of diseases, all characterized by severe anemia for which numerous transfusions had been given, and all of whom developed features of hemochromatosis are presented.
2. Eight similar cases found in the literature are summarized.
3. It is postulated that the hemochromatosis developing in these patients is the end result of the deposition and subsequently irritating action of the excess amounts of iron in the parenchymatous tissues.
4. The underlying anemia and the not infrequent transfusion reactions are thought to act as predisposing factors for the development of exogenous hemochromatosis.
5. The name *Exogenous Hemochromatosis* is proposed for this syndrome.
6. The clinical similarities and dissimilarities and the differences in pathogenesis between exogenous and endogenous hemochromatosis are discussed.

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# HISTOCHEMICAL METHODS APPLIED TO HEMATOLOGY\*

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CHEMICAL cytology deals with the chemical characterization of substances in their natural locations within cells. Its principal aim is to provide a morphologic basis for the understanding of the chemical and functional activities of cells. It attempts to develop histologic staining methods which will have chemical significance and which will characterize and differentiate various kinds of cellular lipids, carbohydrates, proteins, enzymes and inorganic substances. In addition to the use of staining methods, the chemical and physical milieu of cells can be explored by other procedures and techniques, for example, by means of polarized light, ultraviolet light, x-ray diffraction spectra and the electron microscope, to name a few. The present investigation involves the application of a number of methods of chemical cytology to the cells of blood and bone marrow. The results illustrate, we hope, how, by contributing to an understanding of the chemical composition and activity of blood cells, chemical histology can advance the subject of hematology.

The chemical cytology of normal blood and bone marrow has been under investigation for some time in this laboratory. Previous papers have concerned the use of various histochemical procedures for the demonstration of lipids, nucleoproteins, glycogen, acid and alkaline phosphatases, and the phenomenon of metachromasia (Wislocki and Dempsey<sup>1</sup>, Wislocki, Bunting and Dempsey<sup>2</sup>). The present study is a continuation of these lines of investigation. Additional data are presented, including information obtained from a more extensive examination of human blood cells.

The previous observations were carried out mainly on deparaffinized sections of bone marrow. We have now adapted some of the histochemical procedures for use on imprint preparations of bone marrow and on smears of peripheral blood. As a result of these modifications and also from a growing familiarity with the various techniques, our previous observations have been considerably extended. The results of the present study will be presented under the headings of lipids, nucleoproteins, metachromasia, phosphatases and glycogen. In addition to the two papers referred to above, the few other existing investigations in this field will be briefly cited or reviewed.

## MATERIAL AND METHODS

The material for the present study was obtained from 2 young rhesus monkeys and a number of human subjects †. Blocks of marrow from the femurs of the 2

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† The human material was obtained from the Hematological Laboratory of The Pratt Diagnostic Hospital, Boston, Massachusetts, through the interest and courtesy of Dr. William Dameshek.



monkeys were immersed in various fixatives, and from others touch or imprint preparations made on glass slides were placed in identical fixatives \*

Human material consisted of smears of bone marrow and peripheral blood from normal subjects as well as from patients suffering from pernicious anemia, multiple myeloma, Gaucher's disease, lymphocytic, myelocytic and monocytic leukemia, and other pathologic conditions. Peripheral blood was obtained by finger puncture, and marrow by aspiration, usually from the sternum.

For the staining of lipids with sudan black B, blocks of marrow and imprints were fixed in 10 per cent neutral formalin. Subsequently, frozen sections and imprints were immersed briefly in 70 per cent alcohol and stained in a saturated solution of sudan black B for one and seven minutes. Smears of peripheral blood and bone marrow were also fixed by immersion in 70 per cent alcohol for a few seconds, or by placing them for five seconds in a mixture of 10 per cent formalin—1 part, and 95 per cent ethyl alcohol—9 parts (Bailiff and Kimbrough<sup>3</sup>). These preparations were then stained in sudan black B for periods ranging from ten minutes to one hour. They were dipped briefly in 70 per cent alcohol and dried. They could then be counterstained, if desired, with Wright or Wright-Giemsa stain.

Nuclear basophilia was studied by means of the Feulgen reaction following fixation of pieces of bone marrow in Zenker's fluid. For smears of marrow and blood, fixation in 95 per cent alcohol for thirty seconds was utilized and found to be very satisfactory. Light green was used as a counterstain.

For the study of cytoplasmic basophilia, imprints, smears, and blocks of tissue were fixed in Zenker's fluid and stained with eosin and methylene blue. To determine whether cytoplasmic basophilia present in some cells might be due to the presence of ribonucleoprotein, control sections were treated with ribonuclease† before staining them. For this purpose, the sections were digested at a temperature of 60 C for three hours in a 0.1 per cent crystalline ribonuclease solution buffered with sodium barbital to a pH of 6.75.

Another means for the characterization of cellular basophilia consists of measuring the dye-binding capacity of tissue elements photometrically for methylene blue over a range of pH. For this purpose, the tissues were fixed in Zenker's fluid, imbedded in paraffin, sectioned at 5 micra and mounted on glass slides. After deparaffinization, the sections were stained in methylene blue according to the method described by Dempsey and Singer<sup>4</sup> and Dempsey, Wislocki and Singer<sup>5</sup>. The intensity of staining of various substances was determined by measurements made with a photometer (see fig. 1, Dempsey, Bunting, Singer and Wislocki<sup>6</sup>). The figures obtained for light absorption were plotted against pH to form a graph relating dye-binding to the acidity of the staining solution.

Staining of metachromatic substances was carried out on smears, imprints and

\* We are indebted to Mrs. Edith Herman for her assistance in the preparation of the material by the various techniques employed.

† Crystalline ribonuclease was kindly provided by Dr. M. Kunitz of the Rockefeller Institute for Medical Research, Princeton, N. J.

paraffin sections of tissues fixed in 4 per cent basic lead acetate,\* according to the method of Holmgren.<sup>7</sup> Sections cut at 4 micra were stained in a 0.5 aqueous solution of toluidin blue, resulting in a lavender or purple coloration of the metachromatic components of the tissue. Metachromasia observed following this procedure may be due to the presence of mucopolysaccharides, ribonucleoproteins or substances of unknown composition.<sup>8</sup> That attributable to ribonucleoprotein can be identified by exposing control sections to ribonuclease (see above).

For demonstrating the presence of glycogen, smears, imprints and blocks of tissue were fixed in a solution of absolute alcohol, formalin and picric acid (Rossman's fluid). Sections and imprints were then stained by the Bauer-Feulgen method. Since glycogen is dissolved by saliva, control sections so exposed were prepared.

Alkaline phosphatase was demonstrated by the Gomori method,<sup>9</sup> as modified by Dempsey and Deane.<sup>10</sup> Blocks of tissue, smears and imprints were fixed in chilled 80 per cent alcohol. The sections and imprints were then incubated for three and six hours in a solution of sodium glycerophosphate at pH 9.4.

### LIPIDS

*Foreword.* Increasing attention is being accorded sudan black B as a histologic stain for lipids. With this dye, Sheehan<sup>12-19</sup> observed briefly that the leucocytes of human blood became variously tinged. The neutrophilic leucocytes were filled with small, deeply stained granules, whereas the larger granules of the eosinophilic leucocytes appeared to possess merely a surface layer of lipid. The monocytes usually contained lipid granules, while the small and large lymphocytes were always quite free. Myelocytes possessed many sudanophilic granules, and myeloblasts usually, had a small number, while lymphoblasts showed no sudanophilia at all. McManus,<sup>13</sup> studying films of human blood and marrow stained with sudan black B, reported briefly that the cytoplasm of the neutrophilic leucocytes was packed with fine sudanophilic granules, whereas the lymphocytes and monocytes were unstained. Furthermore, granules stained by sudan black B were reported as being present in the cells of the late myeloblast series.<sup>14</sup> Ralph<sup>14</sup> presented an extremely brief report on human blood, examined with sudan black B, to the effect that the granules of neutrophils, eosinophiles and monocytes contained phospholipids as well as lipids extractable with acetone. Lymphocytes and thromboplastids, on the contrary, were said to contain no phospholipids.

Wislocki and Dempsey<sup>1</sup> studied bone marrow and peripheral blood of several young rhesus monkeys in formalin-fixed frozen sections stained with sudan black B. They verified the fact that both neutrophilic and eosinophilic leucocytes of the circulating blood show intense black staining of their granules as do also the corresponding myelocytes of the bone marrow. Lymphocytes of the blood and tissues did not contain lipid particles, but cells which were diagnosed as being

\* Merck's reagent lead subacetate ( $\text{Pb}(\text{CH}_3\text{CO}_2)_2 \cdot \text{Pb}(\text{OH})_2$  mol. wt. 566.52) has been used. The stock must be protected from exposure to air since combination with carbon dioxide results in an insoluble compound. The 4 per cent solution should be made up freshly before using.

monocytes appeared to have a few black particles in their cytoplasm. In megakaryocytes, minute sudanophilic particles were observed, and in blood platelets similar lipoidal dots were encountered. In a subsequent study,<sup>2</sup> evidence was offered suggesting that the sudanophilic material in the cytoplasm of the megakaryocytes might represent mitochondria.

Bailiff and Kimbrough<sup>3</sup> examined normal and abnormal peripheral human blood stained with sudan black B, followed by May-Grunwald Giemsa stain to aid in the differentiation of cell types. They noted that the granules of the eosinophiles were sudanophilic and possessed unstained, clear centers. Basophilic leucocytes, lymphocytes and monocytes were unstained.

From the previous account it will be noted that there is general agreement regarding the behavior of the granular leucocytes, myelocytes and lymphocytes. Of the monocytes, McManus,<sup>13</sup> as well as Bailiff and Kimbrough,<sup>3</sup> claim that they do not stain, whereas Sheehan,<sup>12</sup> Ralph,<sup>14</sup> and Wislocki and Dempsey<sup>1</sup> describe them as being variously tinged.

Excepting Bailiff and Kimbrough, none of the investigators specifically mentions the reaction of the basophilic leucocytes. Because these elements are relatively scarce and with the technic employed difficult to identify, Wislocki and Dempsey<sup>1</sup> and Wislocki, Bunting and Dempsey<sup>2</sup> turned their attention to tissue mast cells. In formalin-fixed frozen sections of several human organs, including uterine cervix and mammary gland, they observed mast cells in which a portion of the normal complement of granules was stained by sudan black. In contrast to the partial or incomplete staining of the granules of mast cells, the granules of tissue eosinophiles were completely and readily stained in similarly prepared formalin-fixed, frozen sections.<sup>2</sup> Meanwhile, Montagna and Noback<sup>15</sup> have reported that sudan black B reveals stained granules in the mast cells of the rat, when applied to frozen sections of tissues fixed in formal calcium-cadmium.

*Observations* The present observations on sudanophilia have been made upon human blood cells from both blood stream and bone marrow. Sudanophilic granules are present in the neutrophilic leucocyte-precursors from the time when specific granules first make their appearance. The sudanophilic granules, staining a grayish brown to black color, are in evidence throughout the series and from their number and size appear to be identical with the specific neutrophilic granulations (fig. 2c). In the myelocyte stage they can be seen overlying the nucleus.

The granules of the eosinophilic leucocytes and of the corresponding myelocytes are sudanophilic. As noted by several investigators, the granules are characterized by a darker, sudanophilic periphery and a clearer, possibly totally unstained, interior (fig. 2a). This peculiarity differentiates their granules from those of the neutrophilic leucocytes.

The basophilic leucocytes in the peripheral blood of several patients with chronic myelogenous leukemia have been examined. Their granules prove to be sudanophilic (fig. 2d), varying in size and ranging in intensity of staining in individual cells from pale gray to deep black. Many of the smaller granules stain

cosinophiles exhibit marked uniformity in regard to depth of staining and size

In the blood of 2 patients in which the monocytes were increased and hence readily identifiable, a variable scattering of sudanophilic cytoplasmic particles was observed (fig 2f). These varied in size and number from a few faint dots up to a dusting of the cytoplasm with quite evident black particles. Since a counter-stain tends to obscure these faint particles, monocytes are best studied without its use.

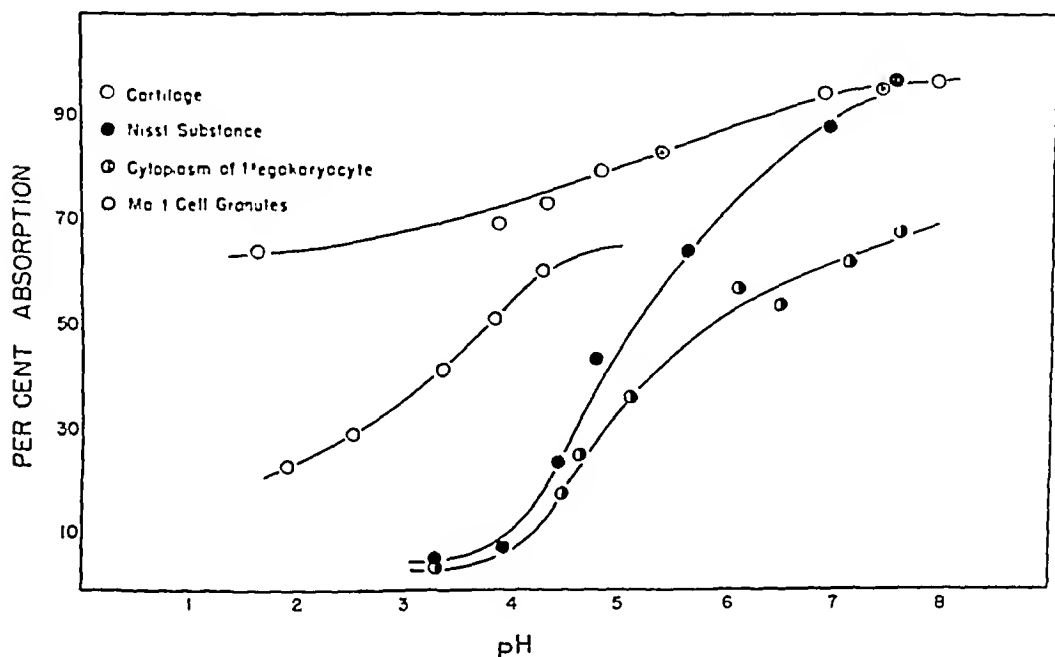


FIG. 1. Curves illustrating and comparing the dye-binding capacity of the cytoplasm of megakaryocytes, mast cell granules, Nissl substance and the hyaline matrix of cartilage. The several tissues were fixed in Zenker's fluid, sectioned, stained with methylene blue, and their dye-binding capacity determined according to a method presented in detail elsewhere (Dempsey et al.<sup>6</sup>). The cytoplasm of megakaryocytes fails to stain below pH 4.0, in this respect being identical with characteristic nucleoproteins such as Nissl substance or cell nuclei. The granules of mast cells and the matrix of hyaline cartilage, on the contrary, exhibit very much stronger acid dissociation, so that staining is not abolished even at pH 1.6 (cf. Wislocki and Dempsey<sup>1</sup> and Dempsey et al.<sup>6</sup>). Their signatures are characteristic of sulfate-containing acid mucopolysaccharides.

In human material, sudanophilia was not observed in either megakaryocytes or platelets. In the megakaryocytes and platelets of the monkey, on the contrary, minute sudanophilic dots have been described,<sup>1</sup> and from their shape, size and number these appear to be mitochondria.<sup>2</sup> In the smears of human material prepared in the manner described, conditions are possibly not so favorable for the staining of these small particles.

We have not encountered lipids in lymphocytes in any of our preparations.

Gaucher cells have not been described as being sudanophilic, but they are rich in cerebrosides (Thannhauser<sup>38</sup>). Because sudan black B appears to have an affinity for a greater variety of lipids than the other sudan dyes currently used in histology, we exposed smears known to contain Gaucher cells to this dye. No sudanophilic

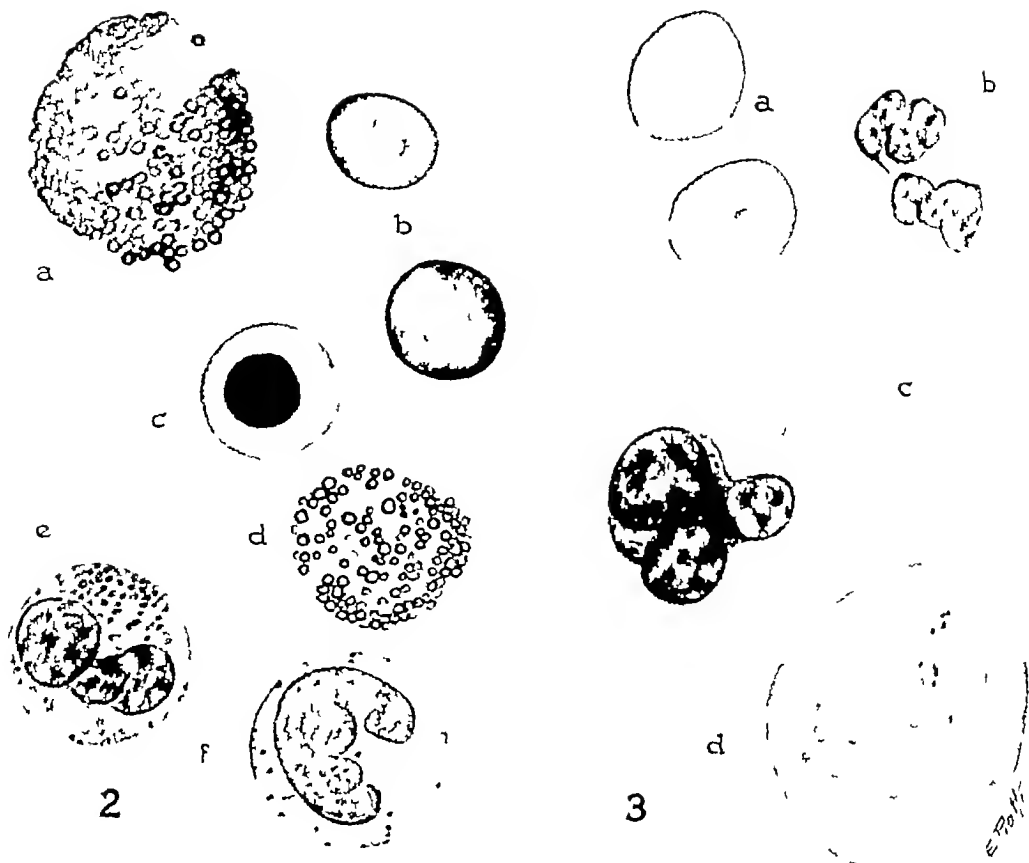


FIG 2 Individual cells from smears of human bone marrow, fixed in 70 per cent alcohol for a few seconds, stained with sudan black B for 10 minutes, and with the exception of d, counterstained with Wright-Giemsa stain All cells in figures 2 and 3 were drawn with a  $\times 10$  ocular and  $\times 90$  objective

a An eosinophilic myelocyte containing granules of uniform size, each composed of a sudanophilic shell surrounding an unstained core

b Two adult erythrocytes showing a dark tinge, superimposed on their normal color, following exposure to sudan black

c Orthochromatic nucleated red blood cell, whose cytoplasm is tinged by sudan black, but not so deeply as adult erythrocytes

d Basophilic leucocyte, stained only with sudan black, exemplifying the variation in size of the granules, and their lesser number as compared with eosinophilic granules Each granule exhibits a dark periphery and light center The granules in occasional basophilic leucocytes are completely blackened more especially those of smallest size

e Polymorphonuclear neutrophilic leucocyte, revealing sudanophilia of its specific granules

f Monocyte illustrating the presence of various sized sudanophilic particles within its cytoplasm

FIG 3 (a, b and c) Individual cells from smears of peripheral human blood and bone marrow fixed for 30 seconds in 95 per cent alcohol, then stained by the Feulgen method which is specific for desoxyribonucleoprotein Counterstained with light green (d) A megakaryocyte from human bone marrow, stained by the Bauer-Feulgen technic

a Two adult red blood cells, one containing a Howell-Jolly body which is stained red and is Feulgen positive, and three blood platelets which are negative

b A polymorphonuclear leucocyte, the nucleus of which is Feulgen positive

c A megakaryocyte whose nucleus also is Feulgen positive It was observed above that blood platelets are Feulgen negative, and it is noteworthy that the cytoplasm of megakaryocytes is also negative

d A megakaryocyte from a section of human bone marrow which was fixed in a solution of absolute alcohol, formalin and picric acid, then stained by the Bauer-Feulgen technic and counterstained with light green Note pinkish-lavender color of cytoplasm and the scattered deeper staining granules in the neighborhood of the nucleus Unlike the Bauer-Feulgen reaction of true glycogen encountered in neutrophilic leucocytes, this reaction in the cytoplasm of human megakaryocytes is not prevented by

material could be demonstrated, and it must be concluded that, unlike some other phospholipids, cerebroside has no affinity for sudan black.

We have observed a faint staining of the red blood cells by sudan black. Adult erythrocytes and the antecedent orthochromatic and polychromatic nucleated red blood cells are diffusely and faintly tinged (fig. 2b, c). The more mature the cell stage, and the longer the preparations are exposed to the dye, the darker the cells become. According to Parpart and Dziemian,<sup>16</sup> the structural meshwork (stromata) of the red blood cells is composed chiefly of lipids and proteins. Among the lipids, phospholipids predominate, while cholesterol makes up the remainder (cf. Hober<sup>17</sup>). Nevertheless, Baker<sup>18</sup> found tests for phospholipids inconclusive in the erythrocytes of the frog and mouse. In view of these considerations, we are not prepared to interpret the observed staining of the erythrocytes by sudan black B.

### NUCLEOPROTEINS

*Foreword.* Two kinds of nucleic acids exist in cells, depending upon the nature of the sugar incorporated into their structure. One, containing a desoxyribose sugar, is called desoxyribonucleic acid, whereas the second contains a ribose sugar and is designated as ribonucleic acid. Nucleic acids are strongly charged compounds and have a strong affinity for basic dyes. Moreover, their basophilia is not destroyed by conjugation with proteins so that the nucleoproteins are also strongly basophilic.

Although nucleoproteins have long been known to display pronounced basophilia and therefore to be among the substances in tissues which react with basic dyes, the positive identification of them and their accurate localization in cells have only recently been accomplished. Three methods have been developed which are of importance in this regard. These are the staining method of Feulgen and Rossenbeck<sup>18</sup> for desoxyribonucleoprotein, the use of ribonuclease developed by Dubos,<sup>19</sup> Brachet<sup>20</sup> and Kunitz,<sup>21</sup> and the utilization by Caspersson<sup>22</sup> of the specific ultraviolet absorption of nucleoproteins. To these should be added a recent method for measuring by photometric means the dye-binding capacity of tissue elements over a range of pH (Dempsey and Singer<sup>4</sup>, Dempsey, Wislocki and Singer<sup>5</sup>).

The Feulgen method consists in the freeing of the aldehyde groups of the desoxyribose sugar by acid hydrolysis followed by the Schiff test for aldehydes. The procedure is apparently specific for desoxyribonucleoprotein (cf. Stowell<sup>23</sup>) and is confined entirely to the nuclei of mammalian cells or to the immediate products of their disintegration or dissolution, as will be shown below. The nucleolus is Feulgen-negative because it is composed of ribonucleoprotein.

The discovery and preparation of an enzyme capable of depolymerizing ribonucleic acid paved the way for the cytologic localization of ribonucleoproteins. Brachet<sup>20</sup> described the ability of ribonuclease to abolish cytoplasmic basophilia in a variety of animal cells, including pancreatic, hepatic and intestinal epithelium, nerve cells and lymphocytes. Wislocki and Dempsey<sup>1</sup> observed the disappearance of cytoplasmic basophilia from erythroblasts, myelocytes, lymphocytes and some other basophilic cells of the marrow following this procedure. More recently, Wislocki, Bunting and Dempsey<sup>2</sup> have noticed a moderate basophilia of

the cytoplasm of megakaryocytes. This basophilia is abolished by treatment with ribonuclease, indicating the presence of ribonucleoprotein in these cells. In addition to various cytoplasmic structures, a number of investigators have noted the disappearance of basophilic staining in the nucleolus after treatment with ribonuclease.

Other basophilic substances, occurring variously in the cytoplasm and in ground substances, may be composed of mucopolysaccharides or of substances of unknown composition. With the photometric method referred to above, which registers the intensity of staining of selected cell constituents over a range of pH, characteristic and specific curves or "signatures" may be obtained for a variety of basophilic substances, including nucleoproteins and mucopolysaccharides.

*Observations.* In the present study we have continued the use of these methods in the study of blood. By the Feulgen method the cell nuclei of human marrow stand out distinctly as reddish purple objects, indicating the presence of desoxyribonucleoproteins (fig. 3). The dense chromatin of the polychromatic normoblasts stains more intensely than the finer chromatin of the younger stages of the red blood cells. The nucleoli are unstained. Imprints of bone marrow stained less well than histologic sections.

Howell-Jolly bodies and blood platelets were noteworthy. The Howell-Jolly bodies were stained by the Feulgen technic, indicating that they are nuclear material consisting of desoxyribonucleoprotein (fig. 3a). Blood platelets, on the contrary, were negative, indicating that they contain no nuclear material (fig. 3a). Voit and Kempa<sup>24</sup> have described a positive reaction for platelets, when stained in thick films, but our observations do not bear out their contention. The delicacy of the reaction in the Howell-Jolly bodies suggests that the reaction would occur similarly in platelets provided that they contained nuclear material. In accord with the negative findings in platelets, it is noteworthy that no Feulgen positive material is encountered in the cytoplasm of megakaryocytes (fig. 3c).

It should be recorded also that neither the basophilic material in the cytoplasm of reticulocytes nor the cytoplasmic stippling of erythrocytes reacts at all, indicating that neither of these structures contains desoxyribonucleoprotein.

The use of ribonuclease on human marrow cells confirms the previous observations of Wislocki and Dempsey<sup>1</sup> on monkey marrow. The cytoplasmic basophilia of the red blood cells precursors, as well as of the basophilic stem cells of the leucocytes, is abolished by the use of this enzyme. This holds true of myeloblasts and promyelocytes, as well as megakaryocytes. The cytoplasmic basophilia of lymphocytes and plasma cells is likewise abolished.

These findings are of interest in view of the recent important discovery that a major function of lymphocytes is to produce globulins which participate in immune reactions (Dougherty and White<sup>25</sup>, Ehrlich and Harris<sup>26</sup>). Consequently the globulin synthesis of the lymphocytes can now be related to the cytoplasmic nucleoproteins which are responsible for the basophilic properties of the cells (Dempsey and Wislocki<sup>32</sup>).

By means of the photometric method, described above, for the characterization of basophilic staining, we have concerned ourselves with the cytoplasmic baso-

philia of megakaryocytes. The cytoplasm of this cell interested us particularly because Wislocki, Bunting and Dempsey<sup>2</sup> had arrived at the conclusion, after some previous uncertainty, that it exhibits a moderate amount of basophilia attributable to ribonucleoprotein. The "signature" obtained by the photometric method confirms this conclusion (fig. 1). The curve for the cytoplasm coincides, in regard to degree of acid dissociation and extinction around pH 4.0, with Nissl substance, a nucleoprotein which fails characteristically to stain below pH 4. For comparison, the graph contains curves for the hyaline matrix of cartilage and the granules of mast cells, both of which consist of strongly acid mucopolysaccharides, the staining of which is not abolished even at pH 1½. Thus we have proof from the use of ribonuclease, as well as from the photometric signature, that the cytoplasm of megakaryocytes contains ribonucleoprotein. We have not succeeded as yet in testing blood platelets with either ribonuclease or by the photometric method.

### METACHROMASIA

Metachromasia is a property, which some basophilic substances possess, of changing the color of certain dyes such as toluidin blue or thionin from blue to red. The principal group of substances exhibiting metachromasia is the acid mucopolysaccharides, but nucleoproteins, after certain methods of fixation, also stain metachromatically. Many of the cells of bone marrow exhibit variably faint metachromatic reactions which are prevented by the use of ribonuclease, a result indicating that the reactions are due to ribonucleoproteins. For example, the cytoplasm of megakaryocytes, following fixation in 4 per cent basic lead acetate, especially in the bone marrow of guinea pigs, is noticeably metachromatic when stained with a 0.5 per cent aqueous solution of toluidin blue, but this staining is prevented by the use of ribonuclease (Wislocki, Bunting and Dempsey<sup>27</sup>).

The granules of the mast cells of the connective tissues are intensely metachromatic due to the presence of an acid mucopolysaccharide, possibly heparin as claimed by Holmgren and Wilander.<sup>33</sup> This reaction is not prevented by ribonuclease (Wislocki, Bunting, and Dempsey<sup>2</sup>) and the granules yield a photometric signature characteristic of acid mucopolysaccharides (fig. 1). The granules of basophilic leucocytes are known to exhibit metachromasia, but we have not tested them photometrically.

### PHOSPHATASE

*Foreword.* The amount and distribution of alkaline phosphatase in various blood cells have been investigated to some degree in a variety of animals. Wachstein<sup>28</sup> has described this enzyme in normal and abnormal cells of human blood and bone marrow, according to him, it is not present in red blood cells, lymphocytes, monocytes or eosinophilic leucocytes of circulating blood, but is present in a variable number of neutrophilic leucocytes. In marrow, staining was uneven and involved neutrophilic leucocytes and occasional nucleated red cells. Megakaryocytes were as a rule negative. Wislocki and Dempsey<sup>1</sup> have described various features of the distribution of alkaline phosphatase in the hemopoietic tissue and blood of



rhesus monkeys, while Deane<sup>29</sup> has reported on its presence in the leucocytes of rats. The last-mentioned investigator used nucleic acid, fructose diphosphate, glucose-6-phosphate and adenylic acid as substrates in addition to glycerophosphate. Recognizable differences in blood cells, following the use of these several substrates, have not been very great.

Wislocki and Dempsey<sup>1</sup> report upon the presence of alkaline phosphatase in variable quantities in the cytoplasm of polymorphonuclear neutrophilic leucocytes, metamyelocytes, myelocytes and lymphocytes. As a rule, the enzyme occurs in both cytoplasm and nucleus. No information appears to exist on the occurrence of phosphatase in basophilic leucocytes of either blood or marrow. However, the granules of tissue mast cells of the rat have been shown to contain alkaline phosphatase by Noback and Montagna<sup>34</sup> and Wislocki and Dempsey.<sup>1</sup> Contrariwise, in human tissue eosinophiles, Wislocki, Bunting and Dempsey<sup>2</sup> encountered none of the enzyme. In keeping with an observation on human marrow (Wachstein<sup>35</sup>), little or no phosphatase was observed in the cytoplasm of megakaryocytes in the bone marrow of the monkey, and insofar as platelets could be evaluated, none was demonstrable.

Only brief references exist to the occurrence of acid phosphatase in the cells of the hemopoietic tissue. Gomori<sup>10</sup> reported the blood cells of all species studied as being negative. Deane<sup>29</sup> refers briefly once to its occurrence in occasional polymorphonuclear leucocytes in the rat. In the granules of the mast cells of rats, both Montagna and Noback<sup>15</sup> and Wislocki, Bunting and Dempsey<sup>2</sup> have described acid phosphatase. In the granules of human tissue eosinophiles the latter investigators have not encountered any.

*Observations.* The present observations concern the demonstration of acid and alkaline phosphatases in both tissue sections and imprints of rhesus monkey's bone marrow with glycerophosphate as substrate. In the case of both enzymes there is far more phosphatase visible in the imprint preparations than in the sectioned material. The procedure for both imprints and sections was identical, except that in the case of the latter, paraffin imbedding was employed. A similar experience has been reported recently by Montagna and Noback,<sup>15</sup> who found that in mast cell granules, far less acid phosphatase was present after imbedding the tissue in paraffin than when paraffin infiltration was eliminated. These authors offer the possible suggestion that the enzyme is partially denatured during infiltration in paraffin at high temperatures.

In the present material, alkaline phosphatase is readily identified in neutrophilic leucocytes, metamyelocytes and myelocytes, being most abundant in the latter. It is diffusely and variably present in both cytoplasm and nuclei. The cytoplasm of the megakaryocytes shows a diffuse faint staining. The nucleated red cells are uniformly negative in both cytoplasm and nuclei.

Imprints of monkey marrow prepared for acid phosphatase reveal an abundance of this enzyme in contrast to its almost complete absence in sectioned material. It is predominantly nuclear in location, staining the nuclei of nearly all of the cells present in the marrow. A few of the cell types can be definitely identified. Myelo-

cytes, in addition to diffuse nuclear and cytoplasmic staining, often exhibit a brownish granulation of their cytoplasm. Metamyelocytes and polymorphonuclear leucocytes are readily identified by brown staining of their nuclei. Nucleated red cells and megakaryocytes are also recognizable by differentiation of their nuclei.

### GLYCOGEN

*Foreword.* Neukirch,<sup>31</sup> in 1910, observed material stained by iodine or Best's carmine method for glycogen in human neutrophilic leucocytes of inflammatory exudates. The material was soluble in saliva and consequently he decided that it must be glycogen or some closely related carbohydrate. Similarly, Stahl, Horstmann and Hilsnitz,<sup>36</sup> utilizing iodine vapor, observed staining of the granules of the neutrophilic metamyelocytes and leucocytes, this they attributed to the presence of glycogen without, however, testing it with saliva.

In human blood platelets Neukirch<sup>31</sup> found central bodies tinged by iodine and stainable with Best's carmine, but these were insoluble in saliva. Stahl, Horstmann and Hilsnitz<sup>36</sup> also encountered iodophilic granules in human platelets as well as a very few, seemingly identical cytoplasmic particles in megakaryocytes. However, they did not test any of these granules for their solubility in saliva.

Wislocki and Dempsey<sup>1</sup> investigated the presence of glycogen in the blood cells of rhesus monkeys by means of the Bauer-Feulgen technic and by an ammoniacal silver nitrate method devised by Mitchell and Wislocki.<sup>30</sup> The diagnosis of glycogen was checked by routine saliva-treated controls. Both methods were successful and positive when applied to appropriately fixed material. Glycogen was identified with regularity in bone marrow in the cytoplasm of neutrophilic leucocytes and metamyelocytes. None was seen in the other cell types including megakaryocytes. Glycogen was also found quite regularly in the rim of cytoplasm of the ordinary fat cells of the marrow. In peripheral blood a positive Bauer-Feulgen reaction occurred solely in the polymorphonuclear leucocytes. No glycogen was demonstrable in the blood platelets.

*Observations.* In the present investigation we have repeatedly observed glycogen in neutrophilic metamyelocytes and leucocytes in smears of human blood and bone marrow after staining with the Bauer-Feulgen method and using saliva controls. Glycogen is minimal in neutrophilic metamyelocytes and increases as the cells mature into leucocytes. It is not present in myeloblasts, myelocytes or the red blood cell series.

On re-examining monkey marrow, glycogen was found quite regularly in the cytoplasm of the fat cells. Moreover, in 1 young rhesus monkey, interstitial glycogen was encountered, although nothing similar was noticed in the marrows of 3 other monkeys.

In peripheral human blood, glycogen was encountered solely in the neutrophilic leucocytes. This finding agrees with those of Wagner<sup>37</sup> who has recently shown by chemical analysis that the granular leucocytes are the only carriers of glycogen in normal human blood.

As reported above, Wislocki and Dempsey found the Bauer-Feulgen and ammoni-

acal silver methods for glycogen negative in the megakaryocytes and blood platelets of rhesus monkeys. In contrast to the monkey, the megakaryocytes of human marrow are faintly Bauer-Feulgen positive, but this material is not digested by saliva (fig 3d). Besides a faint pink color, the cytoplasm of the megakaryocyte contains a variable amount of reddish granular material. The platelets, on the contrary, like those of the monkey, are entirely negative. This finding is consistent with the results of Wagner<sup>37</sup> who identified pentose sugar in human blood platelets but found no glycogen.

Glycogen increases in amount in the neutrophilic leucocytes as they mature, and consequently it characterizes the differentiated functional state of this cell instead of its developmental stages. The glycogen does not appear to be localized or bound in the neutrophilic granules, for, as in other glycogen-bearing cells, it usually shifts by fixation to one side of the cell. Besides glycogen, the neutrophils contain alkaline phosphatase, which is extremely variable in amount and maximal quantity in the myelocytic stages. Alkaline phosphatase is encountered in association with glycogen in various tissues of the body and its presence has been related to the synthesis of glycogen which takes place through the dephosphorylation of hexose phosphate by this enzyme (cf Dempsey and Wislocki<sup>32</sup>). Alkaline phosphatase within the neutrophils tends to be diffusely distributed in the cytoplasm rather than segregated in the neutrophilic granules. In mast cells, on the contrary, in which phosphatase is also abundant, the enzyme is quite definitely localized in the granules.

Confirming Wachstein,<sup>28</sup> we have observed that polymorphonuclear neutrophils which have entered the tissues in inflammatory areas contain increased amounts of alkaline phosphatase. It would be interesting to know whether simultaneously the glycogen content of the neutrophilic leucocytes is altered. Neukirch<sup>31</sup> reports that the neutrophilic leucocytes in exudates contain a far greater quantity of glycogen—as revealed by iodine or Best's carmine stain—than normal leucocytes, but this observation deserves to be re-examined and checked by modern methods.

#### SUMMARY

In table 1 we have attempted briefly to characterize some of the principal blood cell types by means of the various histochemical methods. The data assembled in the table are derived from the present study as well as from the previous papers from this laboratory. The condensed phrases of the table should be referred back to the observations and discussions presented in the text. The spaces which are left blank do not indicate negative reactions but signify merely that critical data are still outstanding.

Besides offering new observations, the present paper summarizes some recent literature on the chemical histology of the cells of blood and bone marrow. The investigation concerns the demonstration and characterization of lipids, nucleoproteins, mucopolysaccharides, acid and alkaline phosphatases and glycogen by a variety of recently developed techniques. It serves to illustrate how histochemical procedures can be utilized to explore the chemical composition and functional activities of cells.

TABLE 1 — *Brief characterization of the principal blood cell types by histochemical reactions for lipids, nucleoproteins, phosphatases and glycogen*

Cell	Lipid (sudan black B)	Ribonucleoprot (cytoplasmic basophilia)	Acid phosphatase	Alkaline phosphatase	Glycogen (Bauer Feulgen)
Myeloblast (Sabin)	Negative	Strongly positive			Negative
Myelocyte	Granules uni- formly posi- tive	Positive	Nucleus posi- tive	Positive in cy- toplasm and nucleus	Negative
Metamyelocyte	Granules uni- formly posi- tive	Negative	Nucleus positive	Positive in cy- toplasm and nucleus	Faintly posi- tive
Polyneut	Granules uni- formly posi- tive	Negative	Nucleus posi- tive	Variably pos in cytoplasm and nucleus	Positive
Eosin Myelo- cytes and Leuco	Granules uni- formly posi- tive				
Tissue Eosin	Granules uni- formly posi- tive	Negative	Negative	Negative	Negative
Baso Leuco	Granules vari- ably positive				
Mast Cells	Granules vari- ably positive	Granules negative	Nucleus neg Granules pos	Nucleus neg Granules pos	Negative
Early Ery thro- blast	Negative	Strongly positive			Negative
Late Erythro- blast	Cytoplasm very faintly tinged	Positive	Nucleus posi- tive		Negative
Normoblast	Cytoplasm faintly tinged		Nucleus posi- tive		Negative
Erythrocyte	Faintly tinged	Negative		Negative	
Megakaryocyte	Minute dots (monkey) None (man)	Moderately positive	Nucleus posi- tive	Cytoplasm faintly pos	Negative (mon- key), Positive (man), Saliva- insoluble
Blood platelets	Minute dots (monkey) None (man)				Negative
Lymphocytes	Negative	Strongly positive		Cytoplasm and nucleus variably pos	Negative
Monocyte	Minute gran- ules			Negative (cit Wachstein)	Negative

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## CLOTTING OF PLASMA AND SILICONE SURFACES

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THE USE of silicone surfaces for blood clotting studies offers new technical approaches to the problems concerned. In their pioneer work, Jaques, Fiddell, Feldsted and Macdonald<sup>1</sup> indicated the possibility that plasma may clot independently of platelet action. They suggest, as others have, that plasma may contain a soluble factor which will initiate clotting. In their studies, the platelet concentrations were lowered by centrifugation at low speeds only to about 3,000 per mm<sup>3</sup>.

We believed that with the use of high speed centrifugation and silicone surfaces blood plasma would remain fluid indefinitely. All the platelets would be removed and even if some should rupture, the thromboplastin released might be expected to be removed by sedimentation in accordance with the work described by Chargin et al.<sup>2, 3</sup>

We followed the venipuncture technic described<sup>1</sup> and clotting was observed at unpredictable intervals. The poor results were apparently due to collapse of the vein with injury to the intima and subsequent liberation of thromboplastic substances. Slowness of withdrawal of blood also increased chances for contamination.

An improved technic was therefore devised which would eliminate tissue trauma insofar as possible. The common carotid artery of a dog was exposed, using meticulous sharp dissection. Saline was flushed over the wound and the artery, after which the wound was draped, leaving only the artery exposed. A special syringe holder (fig. 1) was designed to prevent blood from being forced into the syringe. When the puncture was made, 10 cc. of physiologic saline contained in the syringe were first injected into the artery to wash away thromboplastic substances from the needle. After thirty seconds, the blood was permitted to enter the syringe at the pressure of the blood vessel. It was not necessary to pull on the plunger. The needle and special syringe were next removed and, with the syringe in a vertical position, blood was allowed to flow against the side of the silicone centrifuge tubes. A portion was oxalated for hematocrit and control prothrombin determinations. The tubes were then placed in the rotor of a multi-speed attachment (International Equipment Co.) and spun for varying periods of time at 22-23,000 R P M. After the runs were completed, the tubes were removed and the clear plasma could be seen above the closely packed red cells which were overlaid by a whitish gray layer. To avoid contamination of the supernatant layer with the middle layer, about two thirds of the centrifuge tube was immersed in a mixture of dry ice and alcohol. The red cells, the superimposed gray layer and the lower portion of plasma were frozen solid so that the clear supernatant plasma could be decanted. Samples for prothrombin determinations were taken immediately after centrifuging and at varying periods thereafter.

Our technic eliminated many of the previously encountered erratic results, how-

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ever, it still did not enable us to obtain incoagulable plasma with any degree of certainty. We found that plasma would stay fluid for varying periods—in some runs longer than seventy-two hours at room temperatures. There would usually, however, be some fibrin formed. This occurred in silicone and in glass tubes with about equal frequency, provided centrifugation was carried out for sixty minutes or more. The rotor of the multi-speed attachment accommodates six tubes and the contents of each one would usually give somewhat different results as to time and quantity of fibrin deposited as judged by visible inspection. This fact discouraged us from making quantitative measurements of the fibrin deposits. At the end of twenty-four hours, any fibrin was removed, thrombin was added to the plasma and a clot invariably formed.

Measurements of prothrombin concentration by the two-stage method indicated

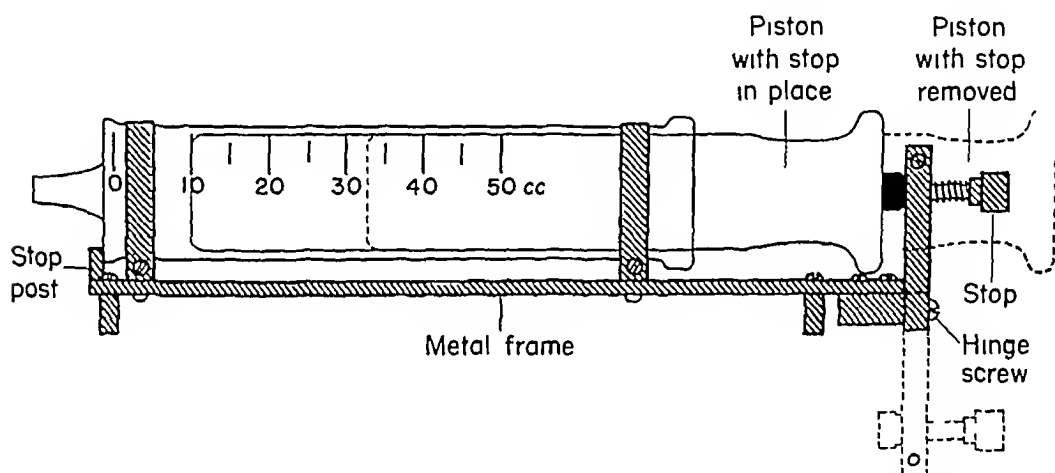


FIG. 1. Special syringe plunger holder for venipuncture. The piston is held in position by the mechanical stop and no blood can enter the syringe until the piston stop is swung to the side.

that this factor does not change appreciably in concentration if the high speed centrifugation is continued for fifteen minutes or more (table 1). Consequently, if thrombin causes the fibrin deposits mentioned above, only a small quantity is involved. It must be kept in mind that rupture of only a few platelets would liberate thromboplastin, which would perhaps not all be removed by centrifugation. Even if it were completely removed, a small amount of thrombin might first be liberated which would eventually form a fibrin deposit. If a soluble plasma factor, not concerned with platelets, initiates clotting, its action must be considered to be extremely feeble. If that view is not accepted, then it must at least be admitted that the soluble factor was sedimented in the centrifuge, or that it requires an activator removed by centrifugation.

Prothrombin analyses showed no significant change in prothrombin concentration. However, evidence for thrombin formation in the silicone plasma was obtained indirectly from Ac-globulin studies. This factor is transferred from plasma type Ac-globulin to serum type Ac-globulin through the action of thrombin.<sup>5</sup> Even small amounts of thrombin will produce an effect which is easily detected. For



example, a quantity of thrombin which will not produce a clot in one hour will markedly affect plasma Ac-globulin activity In the experiments discussed above, it was possible to detect serum type Ac-globulin when the two-stage prothrombin analyses showed no significant change We interpret this as follows a small amount of thrombin was formed by minute traces of thromboplastin of cellular origin This amount of thrombin was so small that the plasma prothrombin concentration seemed not to be altered However, this thrombin was sufficient to transform some plasma Ac-globulin to serum Ac-globulin and also sufficient to form a small fibrin deposit

Fibrinogen was measured quantitatively in only a few experiments for reasons mentioned above The changes in fibrinogen concentration during a typical experi

TABLE I — *Prothrombin and Fibrinogen Concentration in Plasma Kept in Silicone Tubes at Room Temperature*

Hours After Centri- fugation	Per Cent Prothrombin								F†
	5*	10*	15*	30*	60*	120*	240*	300*	150*
0	90	78	100	99	96	100	102	105	85
1	77	57	95	104	—	—	103	115	81
1½	—	—	—	—	—	94	—	—	—
2	80	57	101	104	—	—	—	99	—
2½	—	—	—	—	—	—	100	—	—
3	72	51	109	103	—	—	110	119	—
3½	—	—	—	—	—	92	—	—	7½
5	—	—	—	—	101	—	—	—	—
22	—	—	—	—	68	—	—	—	70

\* Minutes in centrifuge Prothrombin determined on oxalated samples served as control, and this control was considered to have a prothrombin concentration of 100 per cent  
† F = per cent fibrinogen

ment are recorded in table I Immediately after centrifugation the fibrinogen concentration, as measured by methods previously described,<sup>6</sup> was lower than in the oxalate control sample The fibrin was doubtless thrown down as soon as its aggregates formed While the samples stood at room temperature, only small amounts of fibrin precipitated, and at twenty-two hours about 70 per cent of the original fibrinogen remained More fibrinogen disappeared during the centrifugation period than at any other equivalent time interval This suggests the possibility that a few platelets ruptured during the centrifugation

SUMMARY

Blood was collected with special care and centrifuged in silicone treated glass tubes at 22-23,000 R P M for various periods of time Even after prolonged centrifugation, incoagulable plasma could not always be obtained When clots formed, only a small portion of the fibrinogen was represented as fibrin Minute amounts of thrombin cause this fibrin formation No evidence was found to support the view that plasma may contain a soluble factor which can initiate clotting independent of platelet action

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# DETERMINATION OF THE NUMBER OF ERYTHROCYTES, VOLUME OF PACKED RED CELLS, HEMOGLOBIN AND OTHER HEMATOLOGIC STANDARDS IN MEXICO CITY (ALTITUDE 7,457 FEET) STUDY MADE ON TWO HUNDRED HEALTHY PERSONS

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THIS work is a contribution to the knowledge of certain hematologic standards among a group of healthy residents of Mexico City. The standards studied are: volume of packed red cells, hemoglobin in grams per 100 cc of blood, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular diameter, mean corpuscular thickness and sedimentation rate.

Hedin and Blix in 1890 and Daland<sup>1</sup> in 1891 were the first investigators to introduce the use of the hematocrit, that is, the determination by special procedures of the volume of packed red cells in a given amount of blood.

Chapin,<sup>3</sup> Millar,<sup>4</sup> Kennedy and Millikan<sup>5</sup> and Ponder and Saslow<sup>6</sup> believe there exists a 2 to 12 per cent factor of error in the determination of the hematocrit. They found this factor of error when measuring the cell volume by means of other procedures, such as those that include the use of dyes, proteins and tagged erythrocytes containing radioactive iron. But this factor of error, if it exists, does not invalidate the procedure for practical purposes.

The standards investigated in this work are probably of definite value in the study of the anemias. They give a clearer idea of the extent to which one of the most important functions of the blood, hematoses or blood oxygenation, is modified. The red cell count alone does not give sufficient information concerning this function, since there may exist microcytosis or hypochromia or both. Furthermore, the mean corpuscular volume and the mean corpuscular hemoglobin concentration cannot be determined when only the red cell count and the hemoglobin, without the volume of packed red cells, are known.

The results obtained by different investigators are in agreement with the fact that the hematologic standards are the same for all races and economic levels.<sup>7-16</sup> Changes due to age and sex have also been established.

Smith et al,<sup>17</sup> Licknatzky,<sup>18</sup> Acton and Harvey,<sup>19</sup> Haden,<sup>20</sup> Horneffer,<sup>21</sup> Barcroft et al,<sup>22</sup> Cordero,<sup>23</sup> Vergara Lope,<sup>24</sup> Ocaranza,<sup>25</sup> Izquierdo<sup>26</sup> and others found an increase in the number of erythrocytes associated with an increase in the altitude. Licknatzky<sup>18</sup> and Fitzgerald<sup>27</sup> observed an increase of hemoglobin in the same conditions. Hurtado<sup>28-29</sup> and Andresen et al<sup>30</sup> found that the mean corpuscular volume is greater at high altitudes than at sea level. Other climatic factors have no influence, as Walters,<sup>31</sup> Wintrobe<sup>32-33</sup> and Myers and Eddy<sup>13</sup> have shown in their works.

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As the result of numerous investigations, it has been stated that the normal erythrocyte count in Mexico City is in the range of 6,000,000 per cc or more. This statement does not agree with the constant results of red cell counts made in our clinic. Furthermore, the volume of packed red cells, mean corpuscular hemoglobin concentration standards of healthy persons, have never been established in Mexico City and some contradictory reports exist regarding the red cell diameter.

Considering the variation of some of the hematologic standards at different altitudes as well as the above-mentioned facts, it was deemed necessary to study them in Mexico City, because the normal standards found in other countries at sea level cannot be applied in the central plateau of Mexico, which is located at an altitude of 7,457 feet.

The establishment of hematologic standards in Mexico City is necessary not only in order to evaluate correctly the hematologic results in patients, but also to corroborate or contradict the recent discovery communicated by some investigators<sup>28-29</sup> who state that at high altitudes a well defined macrocytosis is present.

#### MATERIAL

One hundred normal females and 100 normal males were selected, with ages ranging from 15 to 31 years in the latter and 14 to 25 in the former. The ages of 14 and 15 were taken as the lower limits, following Osgood,<sup>11</sup> Mugrage and Andresen,<sup>34</sup> Sachs, Levine and Griffith,<sup>35</sup> Goldhamer and Fritzell,<sup>36</sup> Wintrobe,<sup>37</sup> and Williamson,<sup>38</sup> who found them to be the ages during which the stabilization of the number of red cells, hemoglobin and hematocrit begins. Williamson<sup>38</sup> obtained higher values for hemoglobin in males after the age of 20.

Our subjects were selected from different economic levels, particularly among medical and other students, draftees and nurses. All of them were in apparent good health, with well balanced diets, and most of them had lived in Mexico City since birth. A minimum of two years of residence in Mexico City was considered a necessary condition for those not born here. The average height, age and weight were 1.70 meters, 20.9 years and 59.82 kilograms in the males, and 1.58 meters, 18.04 years and 51.83 kilograms in females (table 1).

*Methods and technic.* All the individuals were subjected to a complete clinical examination performed by one of us (J. R. G.) and to a complete survey of their diets. Among 225 persons registered, 25 were discarded because of an existing or recent disease, deficient nutrition or a deficient constitution.

The following studies were performed on each of the selected persons: red blood cell count, volume of packed red cells, amount of hemoglobin in grams per 100 cc, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, sedimentation rate and standard Kahn, Eagle (flocculation test) and AO<sub>3</sub> serologic reactions for syphilis. In addition, determinations of the mean corpuscular diameter were performed in 15 subjects. For these determinations, blood was obtained by venepuncture at the bend of the elbow after a fasting period of eight hours and according to the following technic:

1. Local cleansing with a 2 per cent iodine-alcohol solution.
2. Adjustment of tourniquet for not more than 1 minute so as to avoid alterations of blood concentration and circulation.
3. Collection of 5 cc of blood with B-D needles number 20. The least possible traction was made in drawing the blood. The blood thus obtained was placed in flat-bottom glass tubes containing Paul-

Heller anticoagulant (0.006 Gm. of anhydrous ammonium oxalate and 0.004 Gm. of anhydrous sodium oxalate for 5 cc. of blood) and mixed thoroughly by shaking for 3 minutes

- 4 Collection of 4 cc. of blood for the serologic tests for syphilis
- 5 The tubes fitted with a rubber stopper were kept in a cool place. In every case the determinations were started within the first hour after the blood was taken
- 6 All the equipment used for the collection of blood and for the determination of the standards was cleansed, dried and prepared according to Wintrobe's instructions.<sup>39</sup> Special care was taken to avoid possible personal errors. The work was carried out by specially trained personnel

TABLE I

	No. of Subjects		Mean and Probable Error		Standard Deviation and Probable Error		Mean Deviation	
	Men	Women	Men	Women	Men	Women	Men	Women
Erythrocytes	100	100	5,389,970 ±0.221	5,014,160 ±0.289	3.29 ±0.155	4.37 ±0.202	2.66	3.39
Hematocrit	100	100	51.23 ±0.1685	45.43 ±0.1415	2.59 ±0.123	2.10 ±0.094	2.13	1.14
Hemoglobin	100	100	17.74 ±0.101	15.20 ±0.065	1.51 ±0.072	0.97 ±0.040	1.26	0.75
M.C.V.	100	100	95.85 ±0.4044	91.20 ±0.47	6 ±0.3302	7.01 ±0.330	4.15	5.99
M.C.H.	100	100	32.97 ±0.18872	30.33 ±0.1955	2.82 ±0.1348	2.90 ±0.1388		
M.C.H.C.	100	100	34.62 ±0.17456	33.37 ±0.1213	2.59 ±0.1233	1.88 ±0.089		
S.R.*	100	100	2.31	8.08				
Age	100	100	20.9	18.04				
Height	100	100	1.70 mts	1.58 mts				
Weight	100	100	59.82 kg	51.83 Kg				

\* S.R. = Sedimentation rate

	Range			
	Maximum		Minimum	
	Men	Women	Men	Women
No. of erythrocytes	6,170,000	6,010,000	4,525,000	4,270,000
Hematocrit	58.5	50	45	41.5
Hemoglobin	20.12	17.70	14.4	11
M.C.V.	111	105.5	80	25
M.C.H.	37.6	35	26	29.3
M.C.H.C.	38	37	28	0.5
Sedimentation rate	16	31	0	14
Age	31	26	15	1.46
Height	1.85	1.74	1.54	40.6
Weight	77.2	77.5	42	

**Red blood cell count** Equipment: Pipets, hemocytometers and cover glasses certified by the U.S. National Bureau of Standards, microscopes with X 10 oculars and X 43 objectives, and 0.85 per cent sodium chloride solution used as diluting fluid and prepared with c.p. sodium chloride.

**Method:** the red blood cells contained in a surface of 0.20 sq. mm. (80 small squares) were counted. Both pipet dilutions and red cell counts were made in each case by two different persons. When the difference between the two counts exceeded 250,000 red cells per cm<sup>3</sup>, they were discarded and repeated. The final figure was obtained by averaging the two counts.

**Hemoglobin determination** Equipment: Two Lantz photocolormeters, one with cylindric cells, the other with square cells, filter number 401. The calibration curves of the colorimeters were obtained by determinations of the oxygen capacity in a Van Slyke volumetric apparatus and of the iron content according to Wong's method. Alternating current was used for one of the photocolormeters and direct for the other.

The blood was diluted to 0.5 per cent in 1.0 per cent solution of anhydrous sodium carbonate. Four readings with four new dilutions by two different technicians were made in each case. The final figures were the average of the eight readings, when the difference between those readings exceeded 0.50 Gm of hemoglobin the whole procedure was repeated.

*Volume of packed red cells* Equipment: Wintrobe hematocrit tubes, with an inside diameter of 3 mm and labeled from 0 to 100. Adequate pipets to fill the Wintrobe tubes.

Procedure: The hematocrits were spun at about 3,000 r.p.m. for 30 minutes. To assure a complete packing, they were spun 15 minutes more. Two hematocrits were determined with each specimen of blood and the figure reported in each case is the average of the two findings.

*Sedimentation rate* Equipment: Wintrobe tubes and specially leveled racks to keep the tubes in a vertical position.

Procedure: That described by Wintrobe.<sup>29</sup> Only one reading was made at the end of an hour. Special care was taken to avoid the formation of air bubbles and the shaking of the tubes once placed in the rack.

*Mean corpuscular diameter and mean corpuscular thickness* Venous blood was obtained in 15 individuals, with the same previous conditions and the same careful techniques as the ones described before.

Procedure: The blood with the Paul and Heller anticoagulant was diluted in Simmel isotonic solution (8.2 Gm of NaCl, 0.02 Gm of KCl and 0.05 Gm of NaHCO<sub>2</sub>) with a pH of 7.1. Only anhydric salts were used.

In each case 200 red cell diameters were read on a wet film, with Bausch & Lomb ocular micrometer and a precision screw.

Wet films were chosen to avoid shrinkage of the red cells.

For the mean corpuscular thickness the following formula was employed:

$$MCT = \frac{\text{Mean corpuscular volume}}{\pi \frac{(\text{Mean corpuscular diameter})^2}{2}}$$

## RESULTS

The average red cell count was found to be 5,389,970 per cubic millimeter among the males and 5,014,160 among the females. The complete data are given in table 1. The distribution curves of the red blood cell counts are given in figures 1 and 2.

Hamre,<sup>14</sup> Wintrobe<sup>37</sup> and most of the investigators agree that there is no difference in the number of erythrocytes between the two sexes during childhood, but that a significant difference appears upon reaching the age of puberty. In our series of cases the difference between the two sexes was significant. No difference was found in the number of red cells in relation to age among persons of the same sex (figure 3). No correlation was found between the number of erythrocytes and the socio-economic status. The influence of body surface on the number of erythrocytes is shown in figure 4.

The average amount of hemoglobin per 100 cc of blood was 17.74 Gm in males and 15.20 in females, no age variations being found. Distribution curves, averages, standard deviation, mean deviation, probable error and range are given in figures 5 and 6 as well as in table 1.

In the male group the volume of packed red cells averaged 51.23 cc, among the females, 45.43 cc (figures 7 and 8, table 1). The volume of packed red cells showed no relation to age or body surface (figures 9 and 10).

In the male group the following averages were also found: MCV, 95.85 cμ, MCH, 32.97 γγ, MCHC, 34.62 per cent, in the female group: MCV, 91.20 cμ,

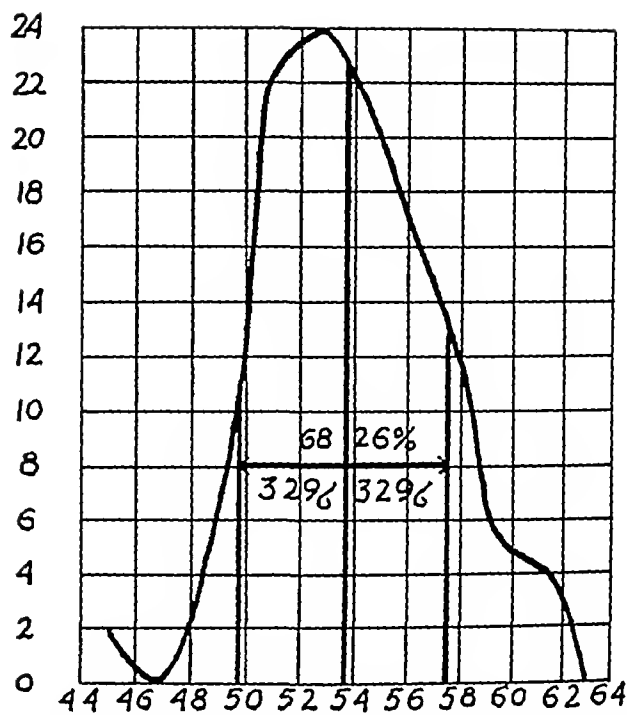


FIG 1. Distribution curve of the number of erythrocytes in 100 healthy men. The standard deviation is 329000 ( $3.29\sigma$ )  
 Ordinates Left-hand scale Number of cases  
 Abscissas Millions of erythrocytes per c. mm

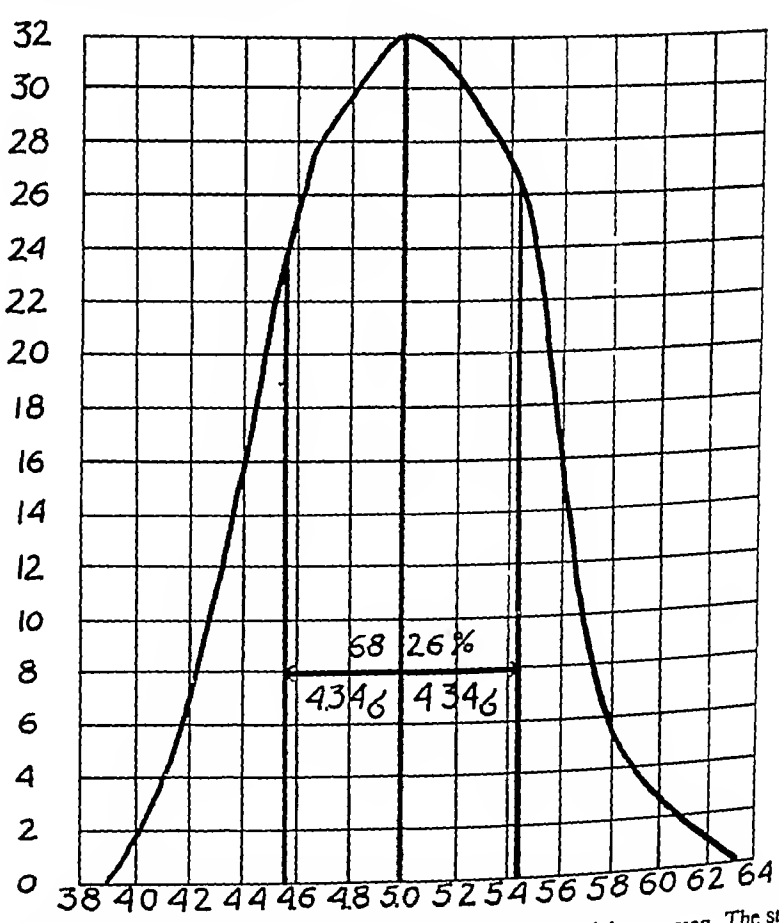


FIG 2. Distribution curve of the number of erythrocytes in 100 healthy women. The standard deviation is 434000 ( $4.34\sigma$ )  
 Ordinates Left-hand scale Number of cases

MCH, 30.33  $\gamma\gamma$ , MCHC, 33.37 per cent. The complete data concerning MCV, MCH and MCHC determinations are given in figures 11, 12, 13, 14, 15, 16 and in table 1.

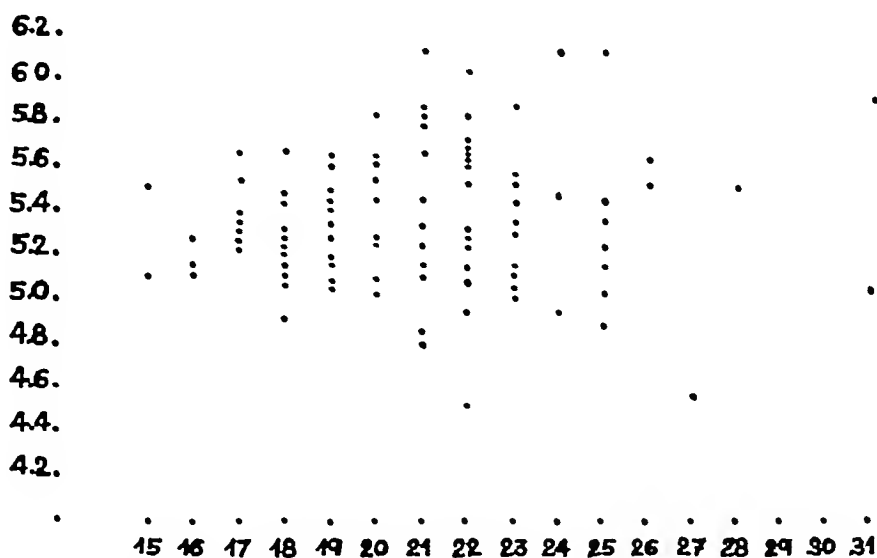


FIG. 3. Study of the number of erythrocytes related to age of 100 healthy men.

Ordinates: Left-hand scale: Millions of erythrocytes per c. mm.

Abscissas: Years of age.

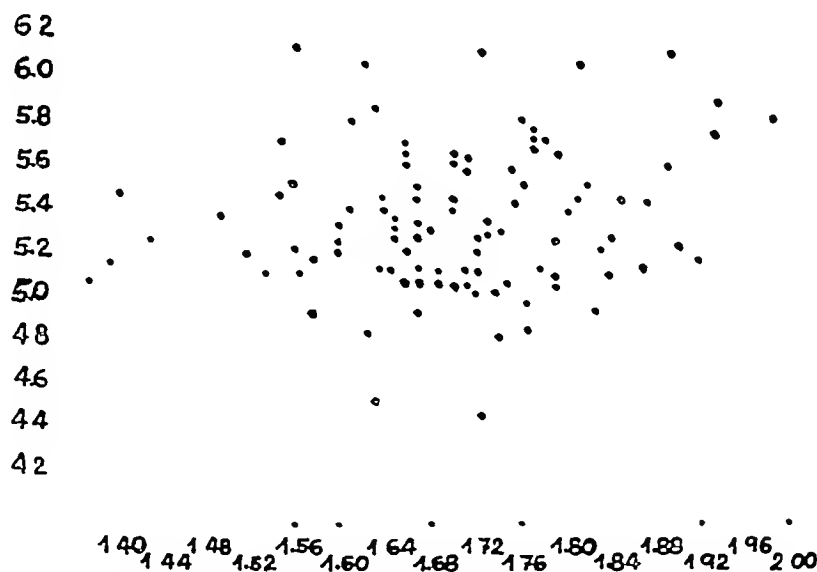


FIG. 4. Study of the number of erythrocytes related to body surface of 100 healthy men.

Ordinates: Left-hand scale: Millions of erythrocytes per c. mm.

Abscissas: Body surface in meters  $2$ .

The relations between the volume of packed red cells, the red cell count and the amount of hemoglobin were studied in each subject. It was noted that in nearly 100 per cent of the cases in which the number of erythrocytes was not within the



HEMATOLOGIC STANDARDS IN MEXICO CITY

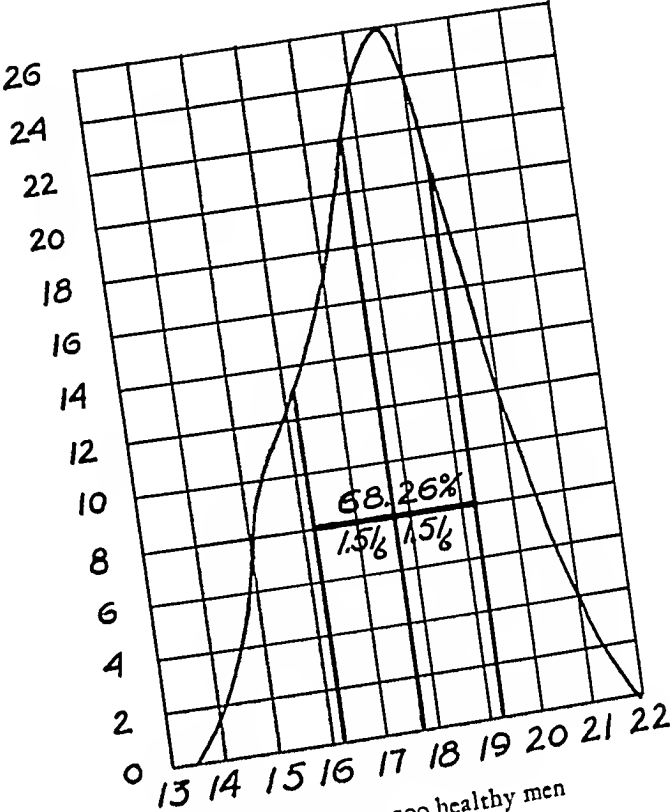


FIG 5 Distribution curve of the hemoglobin in 100 healthy men  
Ordinates Left-hand scale Number of cases  
Abscissas Hemoglobin in Gm

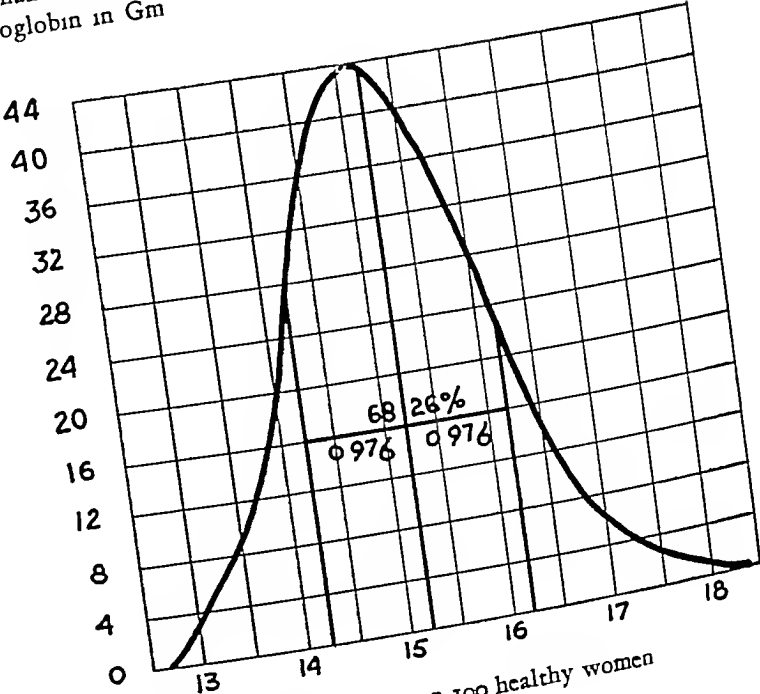


FIG 6 Distribution curve of the hemoglobin in 100 healthy women  
Ordinates Left-hand scale Number of cases  
Abscissas Hemoglobin in Gm

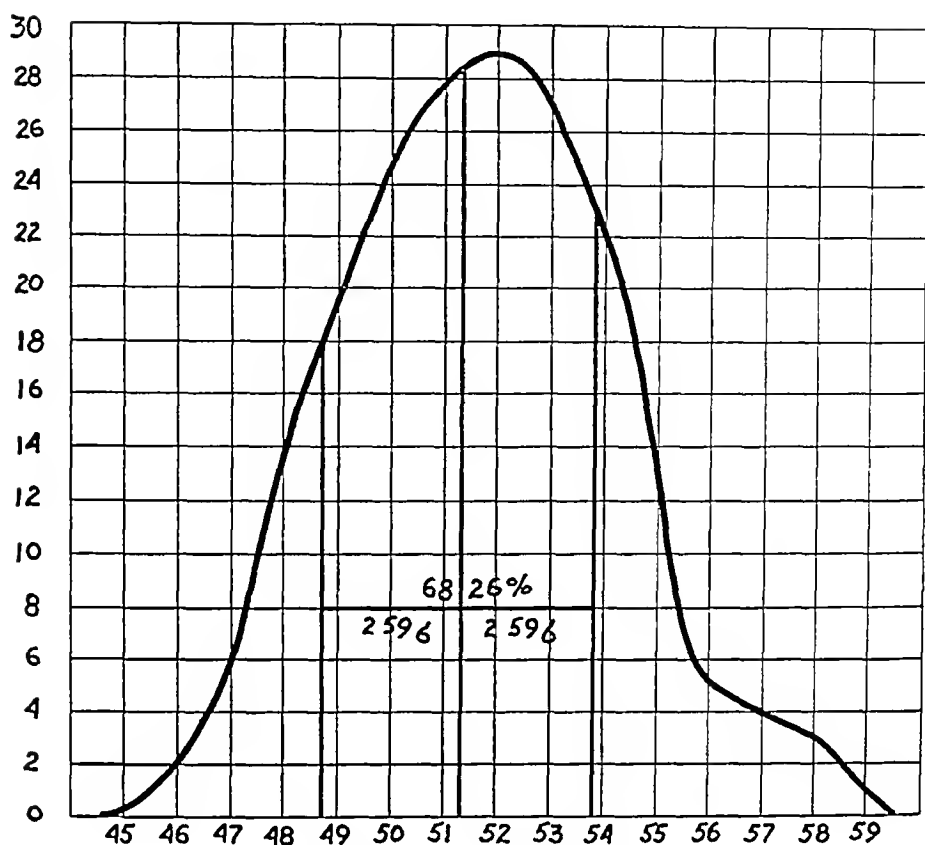


FIG 7 Distribution curve of the hematocrit in 100 healthy men

Ordinates Left-hand scale Number of cases

Abscissas Hematocrit in cc

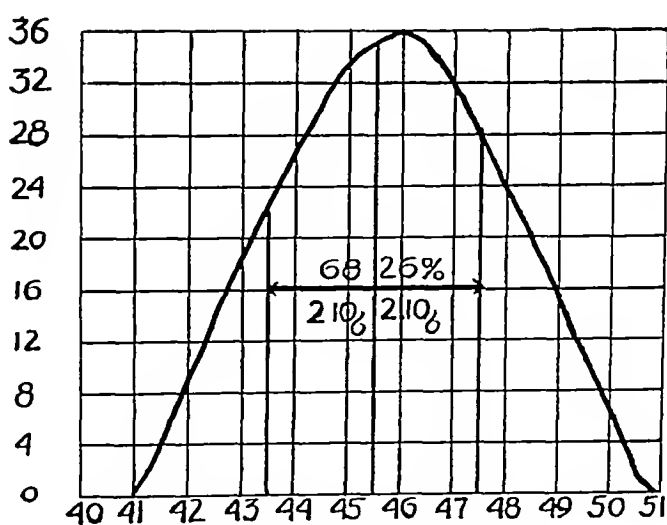


FIG 8 Distribution curve of the hematocrit in 100 healthy women

Ordinates Left-hand scale Number of cases

Abscissas Hematocrit in cc

mean value nor its standard deviation, the volume of packed red cells did not bear out the assumption of anemia or polycythemia suggested by the red cell count alone, since it was found that a higher and a lower volume of packed red cells than normal corresponded to a lower and a higher cell count respectively (figs 17 and



FIG 9 Study of the hematocrit related to age in 100 healthy men

Ordinates Left-hand scale Hematocrit in cc

Abscissas Years of age

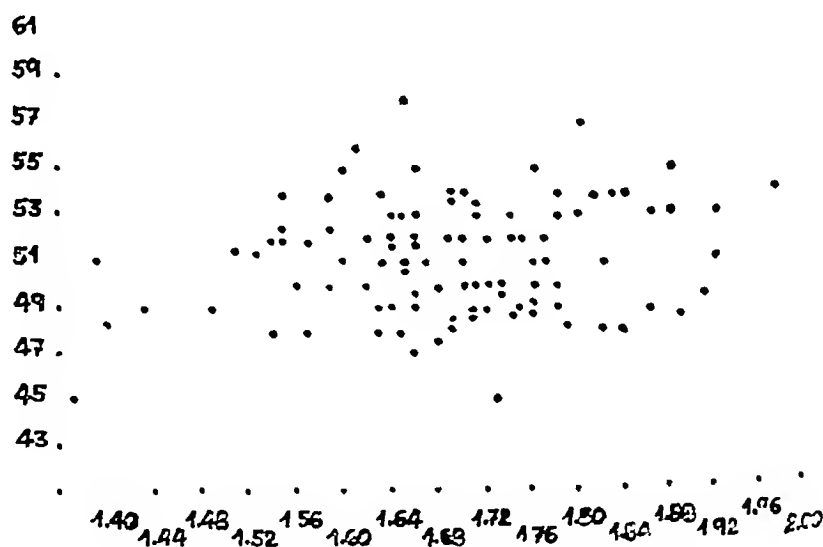


FIG 10 Study of the hematocrit related to body surface in 100 healthy men

Ordinates Left-hand scale Hematocrit in cc

Abscissas Body surface in meters 2

18) Owing to this compensating mechanism, the amount of hemoglobin is kept within normal ranges

The determinations of the amount of hemoglobin, volume of packed red cells and number of red cells per cubic millimeter are also essential to appreciate the distribution of the hemoglobin in the erythrocytes

The mean corpuscular diameter found in the 15 individuals studied was  $8.70 \mu$

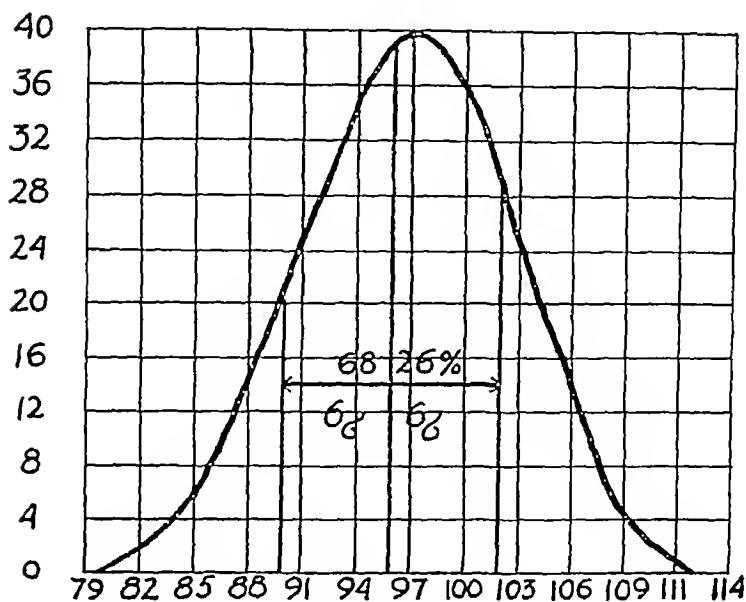


FIG 11 Distribution curve of the M C V in 100 healthy men

Ordinates Left-hand scale Number of cases

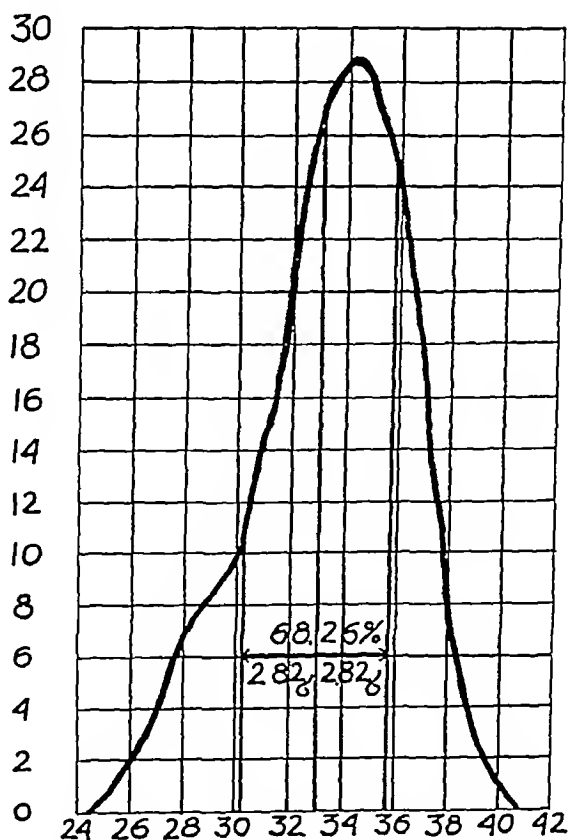
Abscissas Mean corpuscular volume in  $\mu$ 

FIG 12 Distribution curve of the M C V in 100 healthy women

Ordinates Left-hand scale Number of cases

Abscissas Mean corpuscular volume in  $\mu$

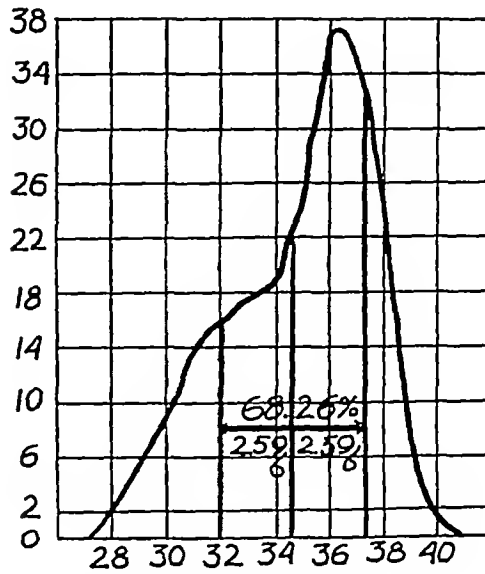


FIG 13 Distribution curve of the MCH in 100 healthy men  
 Ordinates Left-hand scale Number of cases  
 Abscissas Mean corpuscular hemoglobin in g%

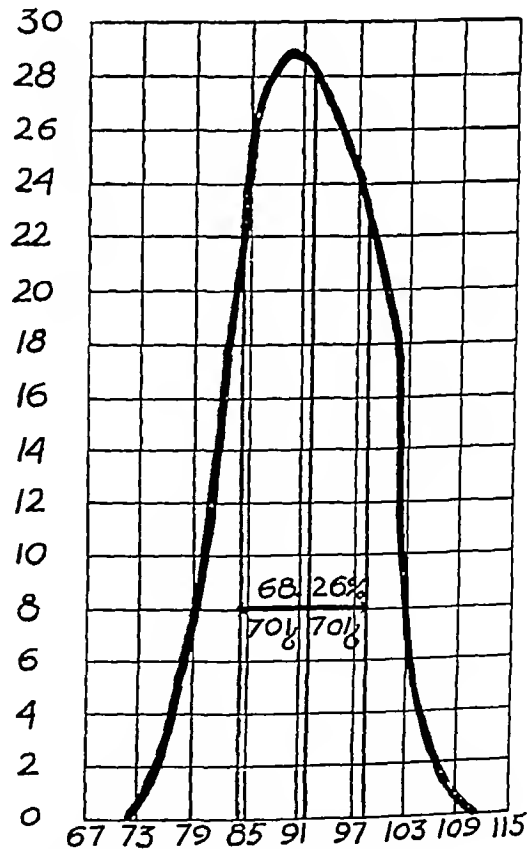


FIG 14 Distribution curve of the MCH in 100 healthy women  
 Ordinates Left-hand scale Number of cases  
 Abscissas Mean corpuscular hemoglobin in g%

Considering the difference of  $0.8 \mu$  to  $1 \mu$  between wet and dry films the equivalent figure on dry films is  $7.80 \mu$ . The mean corpuscular thickness found was  $2.02 \mu$ .

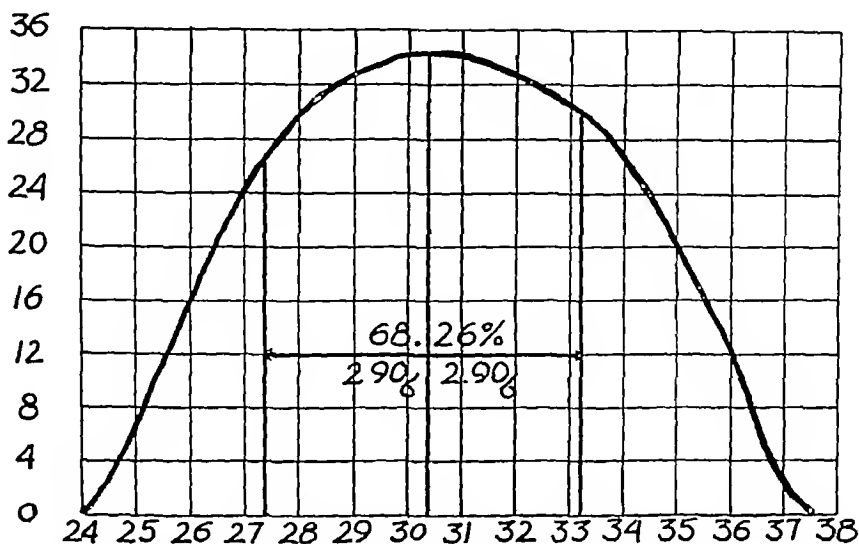


FIG. 15. Distribution curve of the MCHC in 100 healthy men  
Ordinates Left-hand scale Number of cases  
Abscissas Mean corpuscular hemoglobin concentration in per cent

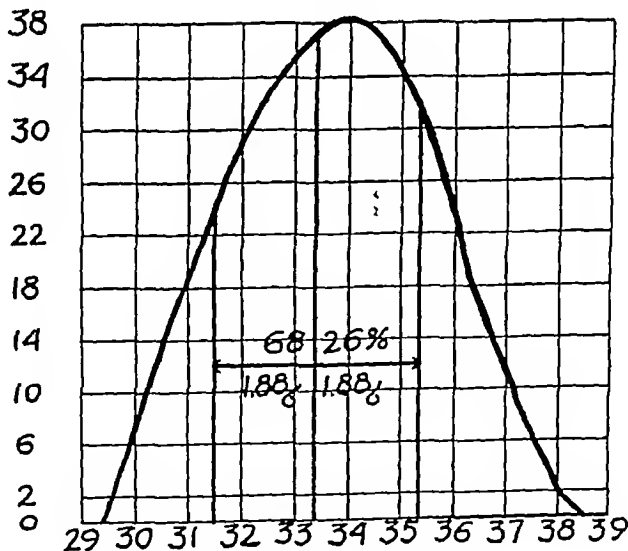


FIG. 16. Distribution curve of the MCHC in 100 healthy women  
Ordinates Left-hand scale Number of cases  
Abscissas Mean corpuscular hemoglobin concentration in per cent

**Sedimentation rate** The average sedimentation rate found in the male group was  $2.31 \text{ mm}$  and it was  $8.08 \text{ mm}$  among females (table 1). There were no cases of foci of infection or of infectious diseases. The menses did not modify the sedimentation rate (figure 19). The sedimentation rates reported are uncorrected according to the volume of packed red cells.

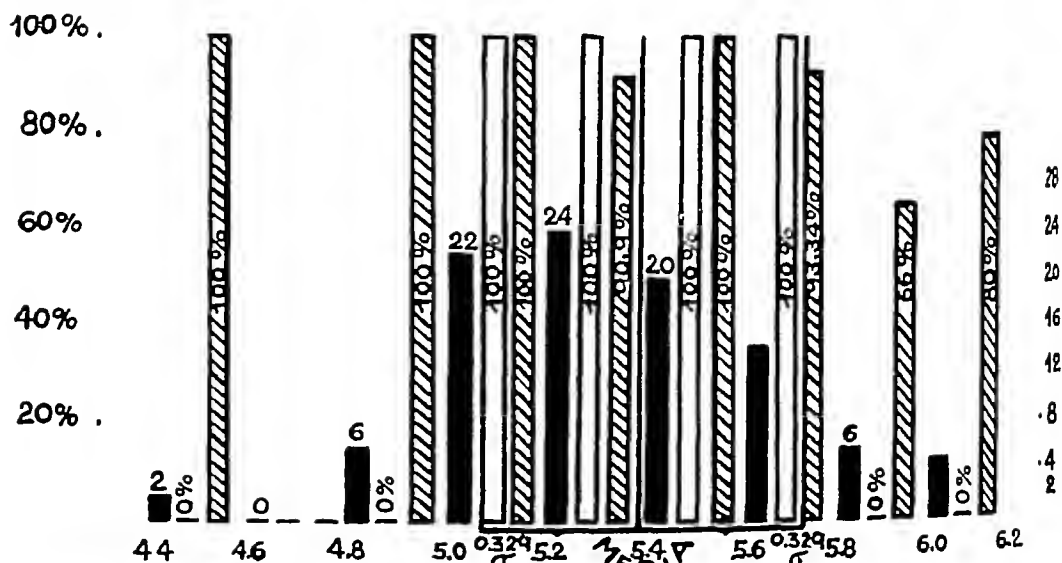


FIG 17 A comparative study between the number of erythrocytes and the hematocrit of 100 healthy men, made in order to judge their normality considering the M C V and the M C H of the erythrocytes

White = number of nonanemic persons judged by the number of erythrocytes Slantlines = number of nonanemic persons judged by the hematocrit Black = number of cases studied in each group

Ordinates Left-hand scale Number of nonanemic persons expressed in per cent Right hand scale Number of cases

Abscissas Millions of erythrocytes per c mm

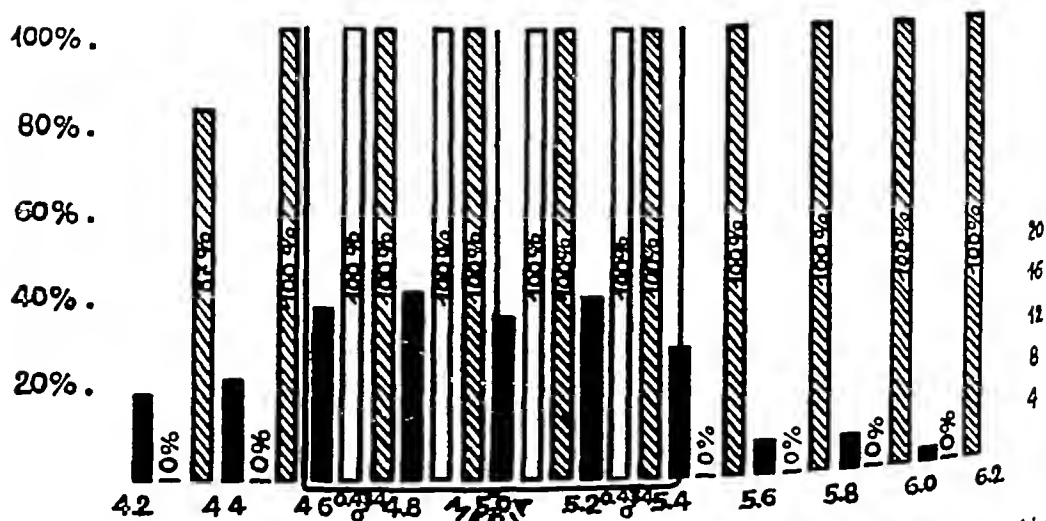


FIG 18 A comparative study between the number of erythrocytes and the hematocrit of 100 healthy women, made in order to judge their normality considering the M C V and the M C H of the erythrocytes

White = number of nonanemic persons judged by the number of erythrocytes Slantlines = number of nonanemic persons judged by the hematocrit Black = number of cases studied in each group

Ordinates Left-hand scale Number of normal cases expressed in per cent Right-hand scale Number of cases

Abscissas Millions of erythrocytes per c mm

## DISCUSSION

In this series of cases it is apparent that the percentage volume of packed red cells and the amount of hemoglobin are slightly above the normal values obtained at sea level. As the red cell counts found are practically the same as those reported at sea level, the MCV and the MCH are higher than at low altitudes. The differences found require a study of the physiologic adaptation of the human body at different altitudes as well as a revision of the literature concerned with hematologic standards in different parts of the world.

As already stated, factors such as age, race, economic levels and most climatic conditions do not modify those standards in the adult.

In Mexico, Cabrera,<sup>40</sup> Izquierdo,<sup>41</sup> and Ocaranza<sup>42</sup> found a slightly diminished red blood cell count among natives of the Teotihuacan valley. A table of the most important investigations performed at sea level and low altitudes is presented in table 2. The investigations made in Mexico City and at other high altitudes are listed in table 3.

The physiologic adaptation of the human body at different altitudes has been studied by a number of workers since the last century. Bert,<sup>62</sup> Viault,<sup>63</sup> Cordero,<sup>23</sup> Vergara Lope,<sup>24</sup> Sánchez Tagle,<sup>68</sup> Douglas et al.,<sup>64</sup> Fitzgerald,<sup>27</sup> Henderson and Haggard,<sup>66</sup> Barcroft et al.,<sup>22</sup> Smith et al.,<sup>17</sup> Ocaranza,<sup>66</sup> Izquierdo,<sup>26</sup> Hurtado,<sup>28-29</sup> Licknatzky,<sup>18</sup> Andresen and Mugrage,<sup>30</sup> Talbott and Dill.<sup>61</sup>

The transient hematologic modifications due to changes in atmospheric pressure are well known. During the first days of an ascent, an increase in the number of red cells has been found by Smith et al.,<sup>17</sup> Licknatzky,<sup>18</sup> Acton and Harvey,<sup>19</sup> Horneffer,<sup>21</sup> Egger,<sup>67</sup> Koppe,<sup>68</sup> Wolfe,<sup>69</sup> Naegeli,<sup>70</sup> and others. When the ascent is rapid, there is an increase of 200,000 to 500,000 erythrocytes per cu mm in half an hour and of a million after 24 hours. Campbell and Hoagland<sup>71</sup> estimate this increase as 5,000 red blood cells for each 1,000 feet. Meyer et al.<sup>72</sup> and others<sup>73</sup> studied the erythrocyte increase in aviators and engineers. Grawits and Gruneberg,<sup>74</sup> Strohl and others<sup>75</sup> studied it in persons placed under low atmospheric pressures artificially obtained in special chambers. The modifications found in the latter study were similar to those found in Alpinists. The immediate increase in the red cell count when ascending is due to an increase in red blood cells secondary to the contraction of the spleen. This is proved by the fact that the increase in the red cell count does not appear in splenectomized animals when ascending. According to Zuntz<sup>76</sup> and Ocaranza<sup>77</sup> there is later an increase in hematopoietic activity, demonstrated directly by bone marrow biopsies and indirectly by the increase of reticulocytes and of bilirubin in the blood as well as by an increase in the whole blood volume, including both plasma and cells of animals and persons living under low atmospheric pressure. Peterson and Peterson<sup>78</sup> and Hurtado et al.<sup>29</sup> under the same conditions have also found a rise in the number of white blood cells.

It should be noted that most of these investigations have been performed in subjects exposed only temporarily to high altitudes, and furthermore, few of the investigators studied all the hematologic and organic changes. Very few of these investigations were made on natives or residents of high altitudes, but, together with ours, they have shown that the increase in the number of red cells is not the



TABLE 2

Authors	Year	Place	No of Cases	Age	No of Erythrocytes	Hb	Hematocrit	M C V	M C H	M C H C
					millions	Gm				
<i>United States</i>										
Sackett <sup>43</sup>	1925	Kansas	15		5 09	16 71				
Osgood <sup>44</sup>	1926	Portland	137	19 30	5 39	15 76	44 64			
Murphy et al <sup>45</sup>	1930	Boston	18		5 35	15 06	44 50			
Wintrobe et al <sup>22</sup>	1929	New Orleans	100	20 30	5 85	15 87	46 50			
Foster et al <sup>46</sup>	1931	New Orleans	115	18 30	5 26	15 63	44 40	85 92	30 07	31 "
Haden <sup>0</sup>	1933	Cleveland	70		4 95	15 34				
Sachs et al <sup>47</sup>	1933	Omaha	100	20 25	5 00	14 93				
Goldhamer et al <sup>26</sup>	1933	Ann Arbor	100	12 17	4 71	11 55				
Helmer et al <sup>48</sup>	1934	Indianapolis	10	20 40	5 58	15 23				
Broun et al <sup>49</sup>	1934	St Louis	23		5 29	16 60	46 57			
Walters <sup>21</sup>	1934	Lawrence	100	20 30	4 84	15 12	46 50	96 50	31 40	37 40
Osgood <sup>12</sup>	1935	Portland	259	14 30	5 42	15 84	44 79			
Peterson et al (Quoted in <sup>14</sup> )	1935	Butte	75		5 17					
Nelson et al <sup>12</sup>	1937	Lawrence	350	18 65	5 11	15 03				
Myers et al <sup>12</sup>	1939	Cleveland	111			15 66				
Hamre et al <sup>14</sup>	1939	Honolulu	137	16 25	5 08	15 10	44 20	86 50	29 20	34 0
Emerson (quoted in <sup>9</sup> )			171		5 44	15 1				
Isaac et al ( <i>Idem</i> )			57		5 08	15 2				
Epstein ( <i>Idem</i> )			42		4 93					
Williamson <sup>25</sup>			140			17 0				
<i>South America</i>										
Orias <sup>50</sup>	1930	Buenos Aires	321			14 28				
Tenconi <sup>51</sup>	1931	Argentina	50		5 30	14 80	43 17			
Parodi (Quoted in <sup>9</sup> )		Argentina	50		5 35	15 4				
Gargiulo ( <i>Idem</i> )		Argentina	944			15 2				
Gargiulo ( <i>Idem</i> )		Argentina	128			17 0				
Meccheri ( <i>Idem</i> )		Argentina	227			15 4				
Hurtado et al <sup>29</sup>		Peru	100		5 26	15 7				
<i>Europe</i>										
Gram et al <sup>52</sup>	1923	Copenhagen	10	20 42	5 45	15 01	46 34			
Heilmeyer et al <sup>53</sup>	1936	Jena	80		5 06	15 90				
Bierring et al (quoted in <sup>14</sup> )	1936	Copenhagen	60		5 07	14 90	44 90			
Dor <sup>54</sup>	1938	Liège		18 40		14 72				
Price-Jones et al (quoted in <sup>29</sup> )		England	100		5 43	14 5				
Jervell et al ( <i>Idem</i> )		Norway	50		5 52	16 2				
Burgi ( <i>Idem</i> )		Switzerland	224		5 00	15 0				
Millet et al ( <i>Idem</i> )		Belgium	50		3 90	13 3				
Jiménez Díaz ( <i>Idem</i> )		Spain	18		4 88					
Komocki ( <i>Idem</i> )		Poland	33		5 84					
Horneffer <sup>21</sup>		Germany	40		4 96	16 0				
<i>India</i>										
Sokley et al <sup>55</sup>	1937	Bombay	121	19 30	5 11	15 37	41 72			
Napier et al <sup>56</sup>	1938	Calcutta	25	20 40	5 05	12 60	42 18	84 93	25 14	29 1
<i>China</i>										
Chia Tu Tien <sup>58</sup>	1931	China	320		5 12					
<i>Philippine Islands</i>										
Chamberlain <sup>16</sup>	1911	Manila	702		5 20	89 60%				
Navarro <sup>57</sup>	1937	Manila	104	19 39	5 16	14 11	43	84	21	27
<i>Australia</i>										
Wardlaw et al <sup>7</sup>	1935	Sydney	26			15 83				
		Central Austr	13			14 95				
<i>Africa</i>										
Licknatzy <sup>18</sup>	1934	Johannesburg	60		5 99	14 54				

only usual and constant feature of the long-standing hematological adaptation to high altitudes. Hurtado et al.<sup>21</sup> among the inhabitants of Morococha, Oroya and Huancayo in the Peruvian Andes found

1. Only a slight increase in the number of erythrocytes compared with the figures found at sea level. In Oroya and Morococha at 3,730 and 4,540 meters respectively above sea level, the averages for red blood cell counts were 5,670,000 and 6,150,000 per c m.

2. Mean corpuscular hemoglobin averages of 33 and 33.9 γγ respectively in the same two places. These figures are slightly higher than those reported at sea level.

TABLE 3

Author	Year	Place	Altitude	No. of Cases	No. of Erythrocytes	Hb
			meters		millions	grams
Cordero <sup>22</sup>	1884	Mexico	2,273	10	5.94	
Angel Gavilón (quoted in <sup>22</sup> )	1888	Mexico	2,273		4.80	
Vergara Lope (quoted in <sup>22</sup> )	1893	Mexico	2,273	38	6.76	
Sanchez Tagle <sup>23</sup>	1896	Mexico	2,273	—	6.06	
Landra <sup>24</sup>	1910	Mexico	2,273	—	6.06	
Ocaranza <sup>25</sup>	1920	Mexico	2,273	—	5.70	15.50
	1920	Mexico	2,273	30	5.94	
Paz (quoted in <sup>22</sup> )		Mexico	2,273	—	6.00	
Del Razo ( <i>Idem</i> )		Mexico	2,273	—	5.50	
Cervera ( <i>Idem</i> )		Mexico	2,273	—	6.00	
Andresen and Mugrage <sup>26</sup>		Denver	1,520	40	5.42	16.54
Fitzgerald <sup>27</sup>		Colorado	1,550	5		15.84
Kuendy et al. (quoted in <sup>29</sup> )		Davos	1,580	—	6.55	17.16
Licknatshy <sup>18</sup>		Johannesburg	1,750	60	5.99	105.4
Hurtado et al. <sup>29</sup>		Huancayo	3,260	10	5.82	
Hurtado <sup>25</sup>		Morococha	4,540	100	6.66	15.93
Morales (quoted in <sup>29</sup> )		La Paz	3,660	200	7.50	
Monge et al. <sup>30</sup>		Oroya	3,730	20	6.88	
Capdehounat et al. (quoted in <sup>29</sup> )		Catavi	3,750	11	6.31	
Barcroft et al. <sup>32</sup>		C. Pasco	4,330	15	7.05	
		C. Pasco	4,330	10		18.85
Talbott and Dill <sup>31</sup>		Quilucha	5,340	6	7.37	22.86

3. A marked increase in the volume of packed red cells and particularly in the mean corpuscular volume 54.1 and 95.2 c μ in Oroya and 59.9 and 97.5 c μ in Morococha.

The increase in the MCV was further confirmed by the finding of greater diameter, thickness and surface area of the red blood cells, as compared with those found at sea level (table 4). A high viscosity of the blood was also found. The blood cell variations mentioned and the slight increase in the serum proteins found by the same Peruvian investigators account for the increased viscosity.

There is a marked difference among the figures found in Mexico by different workers for the mean corpuscular diameter. Cordero reported it to be 5.9 μ, Gavilón, 7.26, and Ochoterena 7.49 (Quoted in reference 25).

The mean corpuscular diameter of  $8.7 \mu$  found in this study is the same one which has been found by Hurtado, considering that our investigation was done on wet films. A difference of  $0.9 \mu$  exists between wet and dry films. These results are slightly above the normal known at sea level.

Andresen and Mugrage<sup>30</sup> in Denver, at an altitude of 1,520 meters, found a volume of packed red cells and a MCV of 48.35 cc and  $93.3 \text{ c } \mu$  respectively for males and 43.22 and  $89.2 \text{ c } \mu$  for females. These values are higher than those reported at sea level, with the exception of those found by Walters.<sup>31</sup>

These modifications of the red cells are probably due to the processes involved in a permanent adaptation to specific conditions found at high altitudes. It is a well known fact that a diminution in the atmospheric pressure and hence a decreased oxygen tension in the lungs is the only constant modification found at such altitudes. The interalveolar tension at sea level is 100, but it can be as low as 40 at high altitudes, and unless the organism compensates for the decrease of interalveolar tension by means of certain physiological processes of adaptation, blood oxygenation cannot be correctly fulfilled at high altitudes.

There is a close interrelationship of all human functions. If a change occurs in the environment, the organism will develop adequate anatomic and physiologic

TABLE 4

	MC Diameter	MC Thickness	MC Surface Area	Blood Viscosity
At sea level	7.48	2.09	137	4.64
In Oroya (3,750)	7.88	1.97	146	8.4
In Morococho (4,540)	7.74	2.08	145	

modifications of its various systems, even of those which are not in direct contact with it.

Physiologic observations show that organisms adapt themselves differently to the same external stimuli, if the length of time that the stimuli exert their action is different. At first, the organism resorts to all its mechanisms at hand, but later, if the external stimuli persist, it replaces those mechanisms with others that are slower to appear but which become permanent and thus more suitable to the needs created by the change.

After rapid ascents there is an increase in cardiac and respiratory rates as well as an increase of the heart output, demonstrated by Barcroft et al.<sup>22</sup> These transitory changes disappear after a few days. Barcroft and his co-workers have also shown (Cerro del Mercado) that after a few weeks of residence at high altitudes there appears an increase in lung ventilation. According to Barcroft,<sup>22</sup> Haldane,<sup>32(p. 355)</sup> and the school of Copenhagen<sup>22 (p. 365)</sup> the increase in lung ventilation is due to the excitability of the respiratory centers, which in turn depends on the decrease of oxygen tension in the blood.

As a result of the hyperventilation there now appears a diminution in the  $\text{CO}_2$  tension and an increase in the oxygen tension with a slight deviation of the blood

pH towards alkalinity. The difference between blood pH at high altitudes and blood pH at sea level is 0.02 to 0.06. Barcroft et al. found at Cerro del Mercado an oxygen consumption one fifth over that at sea level. Murray<sup>52</sup> has shown that there exists a higher oxygen-combining capacity of hemoglobin in persons residing at high altitudes, owing to the increase in hemoglobin alkalinity.

A number of investigators—Douglas et al.,<sup>53</sup> Laquer,<sup>50</sup> Hurtado,<sup>28</sup> Hurtado et al.,<sup>29</sup> and Lozano and Solís<sup>51</sup> among them—have found an increase in blood volume after some weeks of residence at high altitudes. The former used carbon monoxide in their determinations, the others, vital red and Evans blue. The averages found were 6 to 6.8 liters of whole blood and 2.6 liters of plasma, which correspond to 100 to 120 cc of whole blood and 36 to 46 cc of plasma per kilogram. At sea level the normal average of whole blood volume is 5 to 5.5 liters and of plasma 2.8 liters, that is, 85.5 cc of whole blood and 46 cc of plasma per kilogram. A comparison of these figures gives a clearer idea of the increase of total blood volume and especially of cell volume in residents of high altitudes.

Descriptions of special thoracic structures in natives of high regions can be found in the medical literature. Barcroft et al.<sup>22</sup> made radiologic studies of the thorax of persons living in different places and found that the inhabitants at high altitudes have the largest thoracic expansion, the average height of each race having been considered.

Together with all these permanent physiologic and structural changes found in different systems, the red blood cell modifications described before are also seen. There are no phylogenetic observations opposed to the existence of macrocytosis at altitudes above sea level. Although many animals that live at high altitudes have smaller red cells than man's, there are others, like the eagle, which do not show that characteristic. On the other hand, it is true that the disproportion between volume and surface increases with the increase in size of a spherical figure, since they follow a different pattern of growth. This concept, which would explain a handicapped hemoglobin function, cannot be strictly applied to the erythrocyte, since this cell is not spherical but biconcave, and thus its volume is smaller than its surface as compared to the former. Moreover, it has never been proved that the hemoglobin is equally distributed throughout the erythrocyte. In fact, there are theories indicating that the hemoglobin is found specially at the periphery of the red cells (Burke<sup>83</sup> and others<sup>84-85</sup>). Hurtado<sup>29</sup> found the opposite, studying the relations of hemoglobin, red cell area and red cell volume. Perhaps the macrocytosis is due to the diminished oxygen tension in the blood.

The macrocytosis of the red blood cell together with the higher amount of hemoglobin per cell in comparison with the normocyte accounts, from a physiologic point of view, for only the slight hyperglobulia observed by some authors in residents of high altitudes. Owing to the physiologic macrocytosis, there is an increase of the total hemoglobin which favors the interchanges between the alveolar air and the erythrocytes, as well as between the erythrocyte and the body tissues.

The findings described in this paper, as well as the hypothesis<sup>86</sup> sustained in relation with the permanent hematologic adaptation of the organism to high altitudes,

indicate the necessity of further investigations that may or may not substantiate the above-mentioned concepts

### CONCLUSIONS

1 Careful studies of the red blood cells in long-time residents of Mexico City (2,273 meters—7,457 ft) show a slight, although definite, increase in red cell count

2 The percentage volume of packed red blood cells, mean corpuscular diameter and the amount of hemoglobin per 100 cc of blood are higher in Mexico City than at sea level

3 There is an increase in the MCV and in the MCH as a result of the increase in the hematocrit and in the amount of hemoglobin without a definite correlated hyperglobulia

4 The MCHC corresponds to the normal values found by different authors at different altitudes

5 The relation between the hematologic standards studied and factors such as age, sex, diet and body build are in agreement with the findings of other workers

6 The changes mentioned in 2 and 3 have been observed by other investigators working under the same experimental conditions and they differ from those found among temporary residents of high altitudes

7 It is reasonable to assume that the increase of the MCV and that of the amount of hemoglobin are the result of a permanent physiologic process of adaptation to high altitudes, which appears only after a long residence

8 The existence of macrocytosis with increased corpuscular hemoglobin found above sea level possibly favors a better tissue oxygenation as well as other gaseous interchanges

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# THE DEMONSTRATION AND CHARACTERIZATION OF THE ANTI-d AGGLUTININ AND ANTIGEN PREDICTED BY FISHER AND RACE

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WHEN the three-linked-genes theory of inheritance of the CDE/cde (Rh-Hr) blood antigens was proposed by Fisher and Race (1944)<sup>1</sup> some interesting correlations and remarkable predictions were made. On the basis of this theory, new blood antigens designated d and e were postulated in addition to the C, c, D and E which were then recognized. This is easily understood when it is noted that the red cell antigens C and c determined by their respective genes had a reciprocal relationship similar to the M and N antigens. In fact, Levine<sup>2</sup> had earlier described this relationship between C (Rh') and the antigen c (Hr') determined by his original Hr serum. Since D (Rh<sub>0</sub>) and E (Rh'') had no such antithetical relationship to C and c or to each other, separate closely linked loci D and E were assigned to the genes determining these antigens. Because it seemed logical that D and E should likewise have antigens related to them in the same manner as c to C, Fisher predicted that the genes which he designated d and e, should be found.

Following these predictions, Mourant<sup>3</sup> in 1945 reported the finding of a serum which detected an antigen having the specificity required for e. The e antigen having the same antithetical relation to E as c to C indicated that the genes E and e were allelomorphs (i.e. occurring interchangeably at the same locus in the chromosome). This serum, therefore, could be used to determine heterozygosity at the E locus. Although the Fisher-Race theory did not specifically predict more than two allelomorphs for each of the three loci, Callender, Race and Paykoc<sup>4</sup> in 1946 discovered a third allelomorph at the C locus which they designated C\*. This was followed by the finding of an additional allelomorph at the D locus by Stratton<sup>5</sup> in 1946 designated as D<sup>u</sup>. The finding of these two additional allelomorphs was quite compatible with modern concepts of genetics and constituted further evidence for the Fisher-Race theory.

During this period, unfortunately, the predicted anti-d serum remained undiscovered although it had become apparent that this serum would be the most important one for determining heterozygosity of husbands of iso-immunized women. At one time it was thought<sup>6</sup> that the serum reported by Levine and Waller<sup>7</sup> had a specificity suggestive of anti-d. However, this serum was proved to be identical in specificity to the St serum of Race and Taylor (anti-c). At the International Hematology and Rh Conference in Dallas, Texas, 1946, Diamond<sup>8</sup> indicated that he

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had found a serum which contained weak antibodies against the d antigen in addition to anti-c

The present report is concerned with the demonstration of the anti-d agglutinin predicted by Fisher and proof of its specificity. This evidence was obtained by the following (1) Differential agglutination of key red cells of known genotype, (2) quantitative hemolytic studies on such key erythrocytes and (3) determination of specificity of the agglutinin by its reaction with 100 random blood samples

### CASE REPORT

Female, age 26, quadroon, gave a history of having received several transfusions in 1938 while being treated for Paget's disease of the breast. No untoward reactions were recalled. Three previous pregnancies terminated in the delivery of normal infants in 1941, 1943 and 1945. On November 25, 1946 after an apparently normal pregnancy, a female infant weighing 5 lbs. 13 oz. and showing no gross abnormality was delivered at the Charity Hospital, New Orleans. However, typical erythroblastosis developed with marked icterus at eighteen hours. On the second day a hemoglobin determination showed 7.5 Gm. and

TABLE I

Blood From	Group	Antisera			Mother's serum	
		(D) (Rh <sub>0</sub> )	(C) (Rh')	(E) (Rh'')	Saline	Albumin
Father	O	+	—	—	+	+
Child 1	O	+	+	—	+	+
Child 2	A	+	+	—	+	+
Mother	A	+	+	—	—	—

*Subsequently the baby's blood was tested*

Baby	O	+	+	—	+	+
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on the third day, 448 erythroblasts per 100 leukocytes were counted in the infant's blood. The first transfusion of 70 cc. Group O Rh negative blood was given on the day after birth. An additional four transfusions were given as required, the last on the tenth day consisting of 50 cc. of Rh positive blood. The baby was discharged on the twentieth day and has developed normally since that time.

Although the Rh positive erythroblastotic infant was initially given the usual Rh negative transfusions one of the authors (B. W. E.) noted that the mother was Rh positive and suggested the possibility of a rare intragroup isoimmunization. More complete serologic studies were undertaken in the Serologic Research Laboratory of the Southern Baptist Hospital. These studies by one of us (J. W. D.) as given in table 1, showed that the mother's erythrocytes were Group A and contained the C and D antigens but not the E. The father, of Group O, had D but no C or E in the red cells. The infant (and the other two children) gave Group O reactions and their red cells contained antigens D and C but not E. The mother's serum agglutinated the erythrocytes of the father and all three children. Tested against 22 random bloods (13 Rh Positive and 9 Rh Negative) the agglutinins of this serum exhibited no relationship to the M and N types. However, all Rh negative bloods were agglutinated and all but three of the Rh positive blood samples likewise showed agglutination. One additional positive blood showed agglutination in albumin but not in saline suspension. Substantial blood samples were collected from the patient in December 1946 and in March 1947. Serum from these collections were kept frozen at minus 20 C. During the period from December 1946 until May 1947 samples of serum from this case were submitted to many laboratories. All workers agreed that the serum contained an exceptionally strong anti-c (Hr') antibody and many suggested also the possibility of an anti-d antibody. Definite proof of the anti-d however was not forthcoming apparently due to a lack of rare test cells definitely known to be of genotypes negative for c but positive for d.

## METHODS

Studies of the serum of this case begun at The William Buchanan Blood Center in May 1947 were designed to establish the identity of antibodies present by the methods described below. Two techniques were employed to demonstrate agglutination with the key test erythrocytes of known genotype (1) The classic test tube method<sup>9</sup> using 2 per cent saline suspension of red cells and (2) the developing test<sup>10</sup> employing the Coombs<sup>11</sup> antihuman globulin serum. Since the serum from the December bleeding appeared to contain two antibodies differing in reactivity as well as specificity, these two tests were especially useful. The antibody shown to have the specificity of anti-d reacted with both tests while the other antibody with the specificity characteristic of anti-c, reacted with the developing test, but not with the tube method using saline erythrocyte suspension.

The specificity of the two antibody components of the patient's serum was also determined by means of an estimation of their hemolytic action against selected erythrocytes. These studies of hemolytic activity of the serum were made by the quantitative hemolytic technique previously described.<sup>10, 12-14</sup> This method established the net hemolysis resulting from the action of the antibodies present against the corresponding antigens of proper red cell suspensions under the standard optimum conditions over a period of forty-eight hours. Special advantage was taken of the observation made in earlier experiments<sup>12, 13</sup> that the degree of hemolysis was related to the number of antigen antibody combinations available with a given cell and serum. The fact that the two antibodies were present in about equal strength made it possible to interpret the results very clearly.

Although the agglutination and hemolytic tests appeared to establish the specificity of the two antibodies in the patient's serum, it was considered desirable to further check the per cent specificity of the agglutinin component (anti-d) by agglutination of random samples to compare with the specificity of 65 per cent predicted by the calculations of Fisher.<sup>1, 15, 16</sup> In so testing the erythrocytes from 100 random blood samples, the tube method using saline suspension of red cells was employed to bring out the activity of the agglutinin (anti-d) but not the crytagglutinin<sup>\*14</sup> (anti-c) which was inactive against saline suspensions of erythrocytes. As a further precaution, these blood samples were subjected to parallel tests with anti-C, C<sup>x</sup>, c, D, E, e and P. These tests were performed with the Chown<sup>17</sup> capillary technique because of its great economy of serum, especially important with such rare sera as anti-C<sup>x</sup> and anti-c. All the sera so employed were known to be pure and free of other human erythrocyte antibodies.

One of the most important features of this study was the necessity of having available erythrocytes of suitable and definitely known antigenic composition. For example, the test cell of genotype CDe/Cde was of crucial importance in that it could be used to detect anti-d in the presence of the anti-c crytagglutinin found in this serum, without fear that the agglutination might be due to a weak anti-c agglutinin component. Since anti-d serum was not available prior to this case, it was necessary to do family studies to establish the absolute genotype particularly with reference to heterozygosity at the D locus. While individuals of this genotype should not be too rare (about 6.97 per 1000), they would be overlooked and considered as CDe/CDe unless studied in families to show segregation of the Cde chromosome. Fortunately, the genotype CDe/Cde was established in such a family while doing genetic studies with sub-type sera on the random blood samples from the pilot tubes of blood units collected for the Texas City disaster. Of course, the very much rarer (6.4 per 100,000) Cde/Cde could be detected serologically and used to good advantage but because of its rarity was not found in this large series of approximately 1000 blood samples.

## RESULTS

The results of agglutination tests using key erythrocytes designed to differentiate Rh antibodies of different specificities are shown in table 2. The reactions with the

\* *Crytagglutinoids*. Term proposed to designate those antibodies which neither agglutinate saline suspensions of erythrocytes nor give positive blocking tests (saturation of Rh antigen without agglutination in saline). Such antibodies may be detectable by such techniques as the capillary, albumin and serum tests and are uniformly detected by the developing test using a sensitive anti-human globulin serum.

cells shown in this table give strong evidence that an agglutinin of specificity different from anti-C, c, C<sup>u</sup>, D, E or e was contained in the serum of the patient reported in addition to the anti-c cryptagglutinoïd shown by the developing test. The positive reaction with the cell CDe Cde appears to rule in an anti-d agglutinin but does not entirely exclude the possibility of a rare antibody corresponding to an antigen determined by a rare or undiscovered allelomorphous gene of the Rh system or even

TABLE 2

Genotype	Patient's serum tests		Comment
	RBC in Saline	Developing test	
C <sup>u</sup> De/CDe	—	—	rules out anti C <sup>u</sup> , C, D and e
cde/cde	+	+	rules in anti c and/or anti d
cDE/cDE	—	+	rules out anti-E agglutinin rules in anti c cryptagglutinoïd
Cde/CDe	+	+	rules in anti-d agglutinin

\* Identical results were obtained with albumin suspension of RBC

TABLE 3

Probable Genotype	Incidence %	Reactions with antisera						% Positive to Anti-d
		C	c	D	d	E	e	
CDe/cde	32	+	+	+	+	—	+	32
CDe/CDe	15	+	—	+	—	—	+	—
CDe/cDE	16	+	+	+	—	+	+	—
cDE/cde	13	—	+	+	+	+	+	13
cdE/cde	2	—	+	—	+	+	+	2
Cde/cde	1	+	+	—	+	—	+	1
CDe/Cde	1	+	—	+	+	—	+	1
cDE/cDe	1	—	+	+	—	+	+	—
cde/cde	17	—	+	—	+	—	+	17
CDe/cDe	1	+	+	+	—	—	+	—
cDe/cde	1	—	+	+	+	—	+	1
	100							67

an unrelated blood antigen. However, the results from the agglutination studies of 100 random bloods shown in table 3 exclude this possibility. As further exclusion, comparisons with the Lutheran,<sup>4</sup> Kell<sup>18</sup> and Lewis<sup>19</sup> antisera were made and no relationship was found. In this table it will be noted that 67 per cent of random bloods were agglutinated by the suspected anti-d agglutinin. This compares favorably with the 65 per cent predicted by Fisher and Race.<sup>15</sup> However, if the data are examined further, it will be observed that the antigen determined by this serum (65-67 per

cent) has the same antithetical relation to D as M has to N. In other words each specimen is positive for D or d or both but never negative for both.

In figure 1, showing the results of hemolytic tests against CDe/cde and CDe/Cde cells, we obtain a clear confirmation of the presence of both anti-d and anti-c antibodies as well as demonstrating their hemolytic activities. Due to the almost

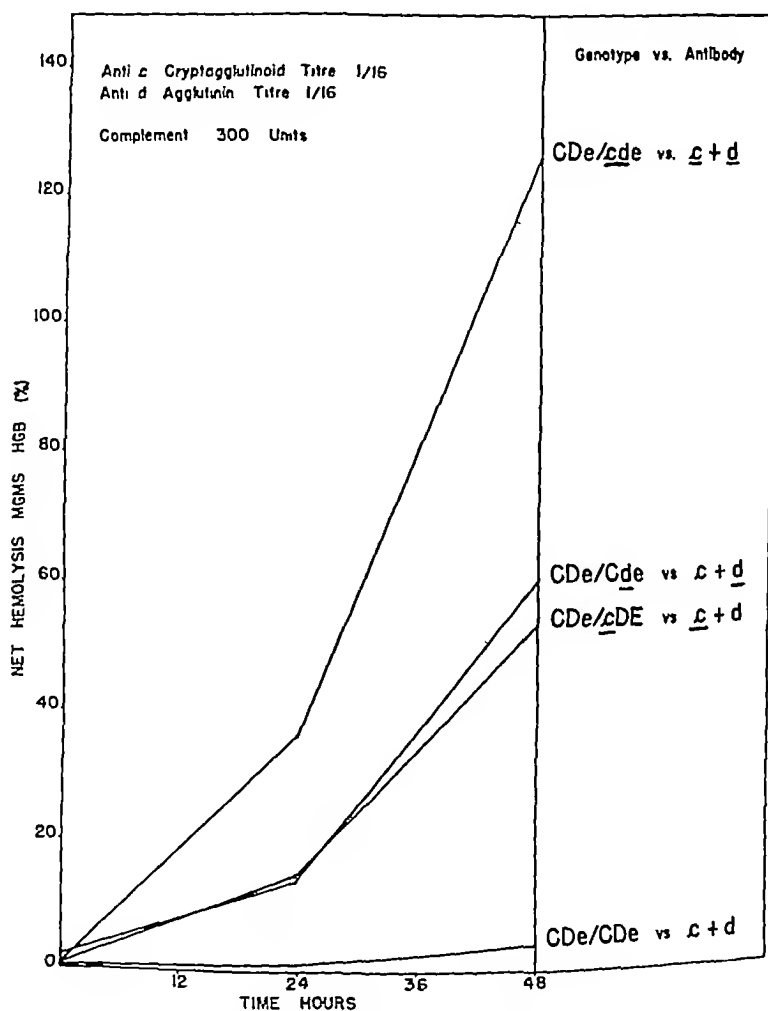


FIG. 1. ANTI-D SHOWN BY QUANTITATIVE HEMOLYTIC TECHNIC

identical titre of the anti-c and d, a unitary relationship between gene, antigen determined thereby, and amount of hemoglobin released by a given strength of antibody was established. This relationship has been previously reported,<sup>12 13 20 21</sup> and will be dealt with in detail elsewhere.<sup>12 13 22</sup>

#### DISCUSSION

The evidence presented in this paper is considered reasonably complete to establish the existence of an antibody having the characteristics predicted by Fisher and Race, namely anti-d. In order to substantiate the validity of such a new antibody and the antigen detected thereby, the following criteria may be considered essential: (1) the antiserum must be shown to give specific antigen-antibody reactions,

(2) it must be shown to differ from previously identified antibodies against human erythrocytes, (3) if more than one antibody is present in the serum it must be possible to recognize their separate effects (4) the percentage incidence (specificity) should be determined on random samples of human blood

The specific nature of the antigen antibody reaction for both components (anti-c and anti-d) was shown by several effects. First, by the use of the developing test the antibodies were shown to be adsorbed on erythrocytes containing the c and d antigens (cDE/cDE and CDe/Cde) and did not adsorb on erythrocytes lacking these antigens (CDe/CDc) as shown in table 2. Since the red cells are washed with saline in the developing test, only immune globulins specifically adsorbed remained on the red cell and therefore constituted a demonstration of the nature of the combination of the antibodies in the patient's serum and the corresponding antigens in the red cells. The specific antibody action of the anti-c and d components of the serum were further demonstrated by their hemolytic activity in the presence of complement as shown in figure 1. Fortunately, in this experiment through the choice of proper erythrocytes, the separate effects of the two antibodies could be observed because of the fact that they were of equivalent strength (titre). Thermal amplitude tests and studies of effect of dilutions of the serum which were done initially eliminated the possibility of panagglutinins, pseudo-agglutinins and cold agglutinins.

The results shown in table 2 indicate that the antibody component (cryptagglutinoïd) demonstrable only by the developing or albumin test was identical in specificity to anti-c. The agglutinin however as shown in tables 2 and 3 did not correspond to any of the available test sera, namely anti-C, C<sup>u</sup>, c, D, E and e. Furthermore, additional tests run in parallel with the anti-Lutheran, anti-Kell and anti-Lewis serums showed no apparent correlation. Finally, comparative tests with anti-A, B and P, and the initial tests with anti-M and N done at the Southern Baptist Hospital ruled out these factors.

From the study of 100 random blood samples (table 3) a specificity of 67 per cent positive reactions was obtained. This compares with the 65 per cent specificity predicted from the calculations of Fisher. This agreement is the more significant when we note the antithetical relation between the reaction of anti-D and the agglutinin component of the serum herein described. These findings indicate allelomorphism of the gene determining the antigen (d) identified by this serum and the gene D.

It is believed that the evidence herein presented constitutes the demonstration and characterization of the d agglutinin and antigen.

#### ACKNOWLEDGMENTS

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Anti-C<sup>u</sup>, Anti-Lutheran, Anti-Kell supplied through the courtesy of Dr. R. R. Race  
Anti-P supplied through the courtesy of Dr. Peter Dahr

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## THE Rh CHROMOSOME FREQUENCIES IN ENGLAND

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D Phil, SYLVIA D LAWLER, M B, B S, and RUTH SANGER, B Sc \*

THIS paper reports the results of testing 1073 samples of blood, from English people, with the Rh antibodies anti-C, anti-C<sup>u</sup>, anti-c, anti-D, anti-E and anti-e. To these are added another 927, tested with anti-C, anti-c, anti-D and anti-E, the results of which have been previously published.<sup>1</sup> Professor Fisher has used the total 2000 to estimate, by his method of maximum likelihood,<sup>2</sup> the frequencies of the Rh chromosomes in England, and has very kindly allowed us to make use of his results.

The discovery<sup>3</sup> of the six elementary Rh antigens and their mutual relationships resulted from a study of the British work of 1943 on the isolation of the seven allelomorphs of the Rh gene. Fisher pointed out that the complex interactions of the four antibodies and seven allelomorphs† known at that time could be most simply understood by assuming that each allelomorph was built up from three elementary antigens, each of which could exist in two alternative or allelomorphic forms. These three pairs of allelomorphic antigens were called C and c, D and d, E and e. A chromosome could carry any combination of the three pairs, it might carry, for example, CDe or cde, etc. Seven of the eight theoretically possible combinations could be identified with the seven allelomorphs already known. The eighth combination, CdE, has still to be isolated, but there is a theoretical reason why it may be very rare.<sup>1</sup>

As an individual has two sets of chromosomes, he has two Rh chromosomes and his genotype may be any combination, for example, CDe/cde. In this case his sex cells, which each contain only one chromosome of a pair, will carry either CDe or cde in equal numbers.

The four known Rh antibodies were thus renamed anti-C, anti-c, anti-D and anti-E. The antibodies anti-e and anti-d which the theory predicted were subsequently discovered,<sup>4, 5</sup> and as a corollary the existence of the predicted antigens e and d were confirmed in a positive manner.

A third alternative to C and c, called C<sup>u</sup>, was found<sup>6</sup> in 1945. It has been isolated in the chromosome combination C<sup>u</sup>De and also C<sup>u</sup>de. Presumably the combinations C<sup>u</sup>DE and C<sup>u</sup>dE exist although they may be very rare.

A third alternative to D and d, called D<sup>u</sup>, was recognized<sup>7</sup> in 1946, and it has been detected in the combinations CD<sup>u</sup>e, cD<sup>u</sup>E and cD<sup>u</sup>e.

Twelve different Rh chromosomes have now been identified, they are CDe, cde, cDE, cDe, C<sup>u</sup>De, cdE, Cde, CDE, CD<sup>u</sup>e, cD<sup>u</sup>E, cD<sup>u</sup>e, and C<sup>u</sup>de. These chromosomes can theoretically give rise to seventy-eight genotype combinations.

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\* Aided by the Australian Red Cross Society, New South Wales Division.

† An apology is needed for our use, in what follows, of the words *genes*, or allelomorphs, as synonymous with *antigens*. To avoid this is cumbersome, and it is possible that there may not be very much qualitative difference between blood group gene and blood group antigen.



## TECHNIC OF THE GENOTYPE TESTS

Owing to the scarcity of the antisera, two of them being unique at the time of the work described, very small volumes are used, approximately 0.008 cc. measured by Pasteur pipet marked with a wax pencil. The tubes used are 7 mm. in external diameter and 50 mm. long. They have a round bottom and are held in 50 hole wooden blocks. Such small volumes of fluid must be delivered nearly to the bottom of the tube.

One volume of serum and one volume of 2 per cent cell suspension in saline are incubated for at least two hours at 37°C. The contents are then gently pipetted onto a slide and read microscopically if agglutination is not obvious to the naked eye.

Although the antisera used were powerful examples of their kind, they were duplicated (excepting the two unique sera anti-C<sup>w</sup> and anti-c). The duplication was performed partly as a check on the results but mainly in the hope of disclosing possible further allelomorphs of C, D, or E.

The 927 bloods of the first series<sup>1</sup> were tested with anti-C, anti-c, anti-D and anti-E and usually two examples of each serum were used.

In the examination of the second series of 1073 bloods, the following antisera were used

anti-C (anti-Rh')	one pure anti C and one mixture, anti-C plus anti-C <sup>w</sup>
anti-C <sup>w</sup>	one serum only
anti-c (anti-Hr)	two examples
anti-D (anti-Rh <sub>0</sub> )	one pure anti-D, one anti-D + anti-D <sup>u</sup>
anti-E (anti-Rh )	two examples
anti-e	one serum only

Anti-A or anti-B were usually removed from the sera by absorption with A<sub>1</sub>B cells of the appropriate Rh genotype, as we had no A<sub>1</sub>B cDE/cDE cells available, the anti-e serum was successfully treated with A<sub>1</sub>B secretor saliva.

In order to avoid selection, we tested blood only from people whose Rh group had not previously been determined, and as far as possible blood of related people was excluded. The samples had, however, often been preselected for their ABO group. Counts on large sections of our results show complete independence of ABO group and Rh genotype. All the blood samples were taken by venipuncture and sent to us in the form of clotted blood.

## RESULTS

Table 1 shows the result of our tests on 2000 blood samples. The first 927 were published in 1946<sup>1</sup> and are here reproduced because Fisher has made use of the combined information given by the two series in his calculation of the chromosome frequencies.

In the second series of 1073 bloods, it will be seen where the use of two further sera, anti-C<sup>w</sup> and anti-e enabled distinctions to be made not possible in the first series. For example, C<sup>w</sup>De/CDe would have been included in the group CDe/CDe of the first series, and cDE/cDE would have been included with the more frequent genotype cDE/cde.

No attempt has been made to include the D<sup>u</sup> distinctions in the tables. There were only two unequivocal examples of D<sup>u</sup> in the 1073 bloods and these have been treated as ordinary D's. Owing to our possessing a pure anti-C<sup>w</sup>, the recognition of the C<sup>w</sup> antigen is easy and can be made in any combination, but there is as yet no pure anti-D<sup>u</sup> serum, consequently D<sup>u</sup> cells can be recognized only in the combination D<sup>u</sup>d, not in D<sup>u</sup>D. D<sup>u</sup>d cells are agglutinated by some anti-D sera (anti-D + anti-D<sup>u</sup>), and not by others (anti-D). This distinction being in part quantitative cannot be made with the certainty characteristic of the other antigen-antibody reactions of table 1.

Table 2 shows the chromosome frequencies calculated by Professor Fisher using

TABLE 1

First Series 927 tested with anti C c D E				Second Series 1073 tested with anti C C <sup>w</sup> -c D-E e			
most frequent genotype in group		observed		most frequent genotype in group		observed	
		absolute numbers	%			absolute numbers	%
CDe/CDe	R <sub>1</sub> R <sub>1</sub>	183	19.74	CDe/CDe	R <sub>1</sub> R <sub>1</sub>	178	16.59
CDe/cde	R <sub>1</sub> r	326	35.17	C <sup>w</sup> De/CDe	R <sub>1</sub> <sup>w</sup> R <sub>1</sub>	12	1.12
CDe/cDE	R <sub>1</sub> R <sub>2</sub>	126	13.59	CDe/cde	R <sub>1</sub> r	354	32.99
cDE/cde	R <sub>2</sub> r	113	12.19	C <sup>w</sup> De/cde	R <sub>1</sub> <sup>w</sup> r	9	0.84
cde/cde	rr	137	14.78	CDe/cDE	R <sub>1</sub> R <sub>2</sub>	138	12.86
cDe/cde	R <sub>0</sub> r	23	2.48	C <sup>w</sup> De/cDE	R <sub>1</sub> <sup>w</sup> R <sub>2</sub>	6	0.56
Cde/cde	R'r	6	0.65	cDE/cde	R <sub>2</sub> r	137	12.77
cdE/cde	R''r	12	1.29	cDE/cDE	R <sub>0</sub> R <sub>2</sub>	29	2.70
CDe/CDE	R R <sub>2</sub>	1	0.11	cde/cde	rr	170	15.84
Cde/Cde	R'R'	0		cDe/cde	R <sub>0</sub> r	19	1.77
Cde/cdE	R'R''	0		Cde/cde	R'r	10	0.93
				cdE/cde	R''r	7	0.65
				CDe/CDE	R <sub>1</sub> R <sub>2</sub>	4	0.37
				Cde/Cde	R'R'	0	
				Cde/cdE	R'R''	0	
				cdE/cdE	R''R''	0	
				cDE/CDE	R <sub>2</sub> R <sub>2</sub>	0	
				CDE/CDE	R <sub>2</sub> R <sub>2</sub>	0	
				C <sup>w</sup> De/CDE	R <sub>1</sub> <sup>w</sup> R <sub>2</sub>	0	
				C <sup>w</sup> De/C <sup>w</sup> De	R <sub>1</sub> <sup>w</sup> R <sub>1</sub> <sup>w</sup>	0	

Four genotypes involving C<sup>w</sup>de could also have been recognized had they occurred

TABLE 2 — Rb Chromosome Frequencies in England

		per cent
CDe	R <sub>1</sub>	40.75499
cde	r	38.86134
cDE	R <sub>2</sub>	14.10870
cDe	R <sub>0</sub>	2.56677
C <sup>w</sup> De	R <sub>1</sub> <sup>w</sup>	1.29296
cdE	R''	1.18819
Cde <sup>1</sup>	R'	0.98349
CDE	R <sub>2</sub>	0.24356

Combinations known (e.g., C<sup>w</sup>de), or believed to exist (e.g., C<sup>w</sup>DE and CdE) have estimates zero, as the data (table 1) do not demonstrate their existence

his maximum likelihood method.<sup>2</sup> By addition to appropriate groups in this table, the frequencies of the genes or elementary antigens are seen to be

C	41.98204%	D	58.96698%	E	15.54045%
c	56.72500%	d	41.03302%	e	84.45955%
C <sup>w</sup>	1.29296%				

Table 3 shows the expected distribution of genotypes based on the chromosome frequencies of table 2. It will be seen that the observed agrees well with the

TABLE 3

1 Genotype		2 Genotype frequencies expected %	3 Phenotype frequencies expected %	4 Absolute num bers expected m	5 Absolute numbers observed m + x	6 $\frac{x^2}{m}$	Series
cde/cde	rr	15 1020	15 1020	302 040	307	0 0815	both
cDe/cde	R <sub>0</sub> r	1 9950	2 0608	41 216	42	0 0149	both
cDe/cDe	R <sub>0</sub> R <sub>0</sub>	0 0659					
cdE/cde	R''r	0 9235	0 9235	9 909	7	0 8540	second
cdE/cdE	R''R''	0 0141	0 0141	151	0	1 2590	second
cDE/cDE	R <sub>2</sub> R <sub>2</sub>	1 9906	2 3259	8 692	12		first
cDE/cdE	R <sub>2</sub> R''	0 3353		24 957	29	0 6550	second
cDE/cDe	R <sub>2</sub> R <sub>0</sub>	0 7243	11 7510	126 088	137	0 9444	second
cDE/cde	R <sub>2</sub> r	10 9657					
cDe/cdE	R <sub>0</sub> R''	0 0610					
cde/Cde	rR'	0 7644	0 7644	130 493	113	2 3450	first
				15 288	16	0 0332	both
CDe/cDe	R <sub>1</sub> R <sub>0</sub>	2 0922	33 8186	362 874	354	0 2170	second
CDe/cde	R <sub>1</sub> r	31 6759					
cDe/Cde	R <sub>0</sub> R'	0 0505					
C <sup>w</sup> De/cDe	R <sub>1</sub> <sup>w</sup> R <sub>0</sub>	0 0664	1 0713	11 495	9	0 5415	second
C <sup>w</sup> De/cde	R <sub>1</sub> <sup>w</sup> r	1 0049					
cdE/Cde	R''R'	0 0234	0 0234	323 429	326	0 0204	first
				0 468	0		both
CDe/cDE	R <sub>1</sub> R <sub>2</sub>	11 5000	12 9478	138 930	138	0 0062	second
CDe/cdE	R <sub>1</sub> R''	0 9685					
cDE/Cde	R <sub>2</sub> R'	0 2775					
cde/CDE	rR <sub>x</sub>	0 1893					
cDe/CDE	R <sub>0</sub> R <sub>x</sub>	0 0125					
cDE/CDE	R <sub>2</sub> R <sub>x</sub>	0 0687	0 0745	0 799	0		second
cdE/CDE	R''R <sub>x</sub>	0 0058					
C <sup>w</sup> De/cDE	R <sub>1</sub> <sup>w</sup> R <sub>2</sub>	0 3648	0 3955	4 244	6		second
C <sup>w</sup> De/cdE	R <sub>1</sub> <sup>w</sup> R''	0 0307					
Cde/Cde	R'R'	0 0097	0 0097	124 383	126	0 0210	first
				0 194	0		both
CDe/CDe	R <sub>1</sub> R <sub>1</sub>	16 6097	17 4113	186 823	178	0 4167	second
CDe/Cde	R <sub>1</sub> R'	0 8016					
CDe/C <sup>w</sup> De	R <sub>1</sub> R <sub>1</sub> <sup>w</sup>	1 0539	1 0793	11 581	12	0 0152	second
C <sup>w</sup> De/Cde	R <sub>1</sub> <sup>w</sup> R'	0 0254					
C <sup>w</sup> De/C <sup>w</sup> De	R <sub>1</sub> <sup>w</sup> R <sub>1</sub> <sup>w</sup>	0 0167	0 0167	0 179	0	0 7624	first
				171 563	183		

TABLE 3—*Continued*

1 Genotype	2 Genotype frequencies expected %	3 Phenotype frequencies expected %	4 Absolute num bers expected m	5 Absolute numbers observed m + x	6 $\frac{x}{m}$	Series
CDe/CDE R <sub>1</sub> R <sub>2</sub>	0 1985	0 2033	2 181	4		second
Cde/CDE R <sub>1</sub> R <sub>2</sub>	0 0048					
C <sup>w</sup> De/CDE R <sub>1</sub> <sup>w</sup> R <sub>2</sub>	0 0062	0 0062	0 067	0		second
CDE/CDE R <sub>2</sub> R <sub>2</sub>	0 0006	0 0006	0 006 1 948	0 1		second first
		99 9999	1999 998	2000		
Sum of 10 smaller classes			10 237	11	0 0569	

$$\chi^2 = 8.2443 \quad 17-8$$

$$m = 9 \quad R = 0.5$$

expected distribution. Columns 1 and 2 show the 36 genotypes, and their expected frequencies, that result from combinations of the 8 chromosomes of table 2. Those genotypes joined by brackets could not be distinguished serologically. Column 3 shows the frequencies expected of the 20 phenotypes distinguishable with the 6 sera used in the second series. Column 4 shows the absolute numbers expected in the 26 observational classes in the two series. Column 5 shows the numbers observed in these classes. The 6 observational classes additional to the 20 phenotypes arise because of the absence from the first series of 2 of the sera used in the second. For example, cDE/cdE and cDE/cde are distinct in the second series, but the 12 cDE of the first series were indistinguishable (in the absence of anti-e) and must therefore be placed in a separate group.

In the calculation of  $\chi^2$  shown in column 6, the 10 smaller classes have been pooled and are shown at the foot of the table.

#### GENETIC BASIS OF THE Rh GROUPS

Fisher suggested that the simplest genetic basis of the allelomorphous antigens, which he had demonstrated, would be a system of three closely linked genes. If the classic conception of a gene is adhered to, this seems to offer the only easy explanation of the observed facts. The alternative is the postulation of a new kind of gene, possessing three subgenes each capable of substitution by allelomorphs.

It seems wiser to conform to the traditional ideas, particularly since, as Fisher has pointed out,<sup>1</sup> there is some suggestive evidence for the occurrence of crossing over. This evidence will now be reviewed in the light of the results described in this paper. In the English population the common chromosomes are CDe, cde and cDE. The three resulting heterozygotes, CDe/cde, CDe/cDE and cDE/cde could give, if crossing over occurred, Cde and cDe, CDE and cDe, and cdE and cDe, respectively. If these rarer chromosome combinations did arise in this way, the

frequency of cDe would be expected to equal the combined frequencies of Cde, CDE and cdE. It will be seen from table 2 that  $Cde + CDE + cdE = 2.41524$  per cent while  $cDe = 2.56677$  per cent. In view of the sampling errors, this agreement is very close. The remaining combination CdE (Ry) would not be produced by a single cross-over from the common heterozygotes. It would require for its production a cross over occurring in a heterozygote involving a rarer chromosome, for example, CDe/cdE. This offers a rather satisfactory explanation of the apparent absence of CdE which is otherwise puzzling.

Fisher has suggested that the order within the chromosome is such that C lies between D and E. The reason for this view is that the frequency ratio of cdE to cDE/cde which represents a cross over between D and E, is considerably larger than the ratios of Cde to CDe/cde (cross over between C and E) and of CDE to CDe/cDE (cross over between C and E). These frequency ratios based on the results of the 2000 bloods are as follows

D—E	108	
C—D	030	} 051
C—E	021	

While this supports Fisher's view that the D—E distance is the greatest, yet the sum of the distances C—D and C—E does not correspond at all well with the total distance D—E. This can be explained by the fact that in the first series cdE was by chance too frequent, compared with a much larger series tested by Stratton<sup>9</sup> with sera capable of detecting cdE. If the calculations are based only on the 1073 of the second series, the distances show remarkable agreement, thus

D—E		074
C—D	034	} 072
C—E	038	

The results obtained by Wiener, Zepeda, Sonn and Polivka<sup>8</sup> in their tests on 99 Mexican Indians, lend support to the theory of crossing over as the cause of the rarer combinations in a population. The only frequent chromosomes in this population were CDe and cDE, and the only common heterozygote CDe/cDE. This, according to the theory, should result in the chromosomes CDE and cDe having approximately equal frequencies in such a population. The chromosome frequencies calculated from this small sample were CDE 3.3 and cDe 1.9.

It should be pointed out that no attempt is made to use the hypothesis of crossing over to explain the different racial distributions of the common chromosomes. It does however afford an explanation of the occurrence of the rarer chromosomes in a population in which the frequencies of the commoner combinations are already known.

#### SUMMARY

The results are reported of testing 1073 English bloods with the Rh antibodies anti-C, anti-C<sup>w</sup>, anti-c, anti-D, anti-E and anti-e. The results of another series of 927 bloods, already published, are here reproduced. The total of 2000 bloods has

been used by Fisher to estimate, by his method of maximum likelihood, the Rh chromosome frequencies in England. The estimates are CDe 40.75 per cent, cDe 38.86 per cent, cDE 14.11 per cent, cDe 2.57 per cent, C<sup>o</sup>De 1.29 per cent, cdE 1.19 per cent, Cde 0.98 per cent, and CDE 0.24 per cent.

A brief account is given of the three pairs of alternative antigens shown by Fisher to be the basis of the Rh blood groups. Fisher's interpretation must now be considered as established beyond doubt. A possible genetic basis of these related antigens is discussed.

#### ACKNOWLEDGMENTS

We are deeply indebted to Professor Fisher for many reasons, but we should particularly like to acknowledge his kindness in allowing us to publish the results of his calculations of the chromosome frequencies.

For the antisera used in the investigations we are indebted to the following: Doctors E. F. Aubert, Sheila Callender, D. S. Dick, R. J. Drummond, Mr. I. Dunsford, Doctors A. J. McCall, Brenda Morrison, J. Murray, E. Wordley and R. A. Zeitlin.

We also thank Dr. H. F. Brewer of the London Red Cross Blood Donor Service, and Dr. J. F. Loutit of the National Transfusion Service, for providing us with large numbers of blood samples.

We also wish to acknowledge the assistance of Dr. Marjory N. McFarlane in the earlier part of this investigation.

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# DISTRIBUTION OF THE Rh TYPES IN SÃO PAULO (BRAZIL)

By F OTTENSOOSER, M D , P H D , C S LACAZ, M D , H C FERREIRA, M.D,  
AND O MELLONE, M D

THE FREQUENCY of Rh positives and Rh negatives in the white population of São Paulo<sup>1-3</sup> is about the same as that observed in the United States and England. In the present publication we deal specifically with the frequency of the eight types of the Rh factor found in São Paulo.

The anti-Rh<sub>0</sub> serum (85 per cent) was selected by ourselves in São Paulo, but both anti-Rh' (70 per cent) and anti-Rh'' (30 per cent) were obtained in New York (Blood Donor Service). We used the technic prescribed by Levine<sup>4</sup> incubating from 30 to 60 minutes in a water bath, centrifugation, and reading the re-

TABLE I

Classes	Anti Rh serum				Anti Rh serum			
	Rh <sub>0</sub>	Rh'	Rh''	Types	Rh <sub>0</sub>	Rh'	Rh''	Types
W	—	—	—	Rh neg	+	—	—	Rh <sub>0</sub>
U	—	+	—	Rh'	+	+	—	Rh <sub>1</sub> (Rh'e)
V	—	—	+	Rh''	+	—	+	Rh(Rh'e)
UV	—	+	+	Rh'Rh''	+	+	+	Rh <sub>1</sub> Rh <sub>2</sub> (Rh'eRh'e)

cell sediment macroscopically and microscopically. That human anti-Rh agglutinins act better at 37 C than at lower temperatures was shown by Levine, Burnham and Katzin.<sup>5</sup>

Using these three anti-Rh sera we could identify eight types of the Rh factor,<sup>25</sup> shown in the scheme by Wiener, Sonn and Belkin,<sup>6</sup> which has been used throughout the United States (table 1).

A very different nomenclature has been accepted in England,<sup>7-10</sup> which makes the subject somewhat more complicated. Until an international nomenclature<sup>11</sup> has been adopted we intend to continue using the nomenclature proposed by Wiener.<sup>12</sup>

Table 2 presents the geographic distribution of Rh types among various populations, including our results in São Paulo (Brazil). It is quite obvious that the frequency of the different types varies considerably according to race. In our statistics a few Negroes and mulattoes were omitted. Unfortunately the anti Rh serum of Levine<sup>5</sup> was not available to us.

From Hospital das Clinicas, University of São Paulo, Brazil

The distribution of the Rh types among the white population of São Paulo (Brazil) is

Rh negative (cde)	15.2 %	Rh <sub>0</sub> (cDe)	5.8 %
Rh' (Cde)	1.4 %	Rh <sub>1</sub> (CDe)	55.2 %
Rh'' (cdE)	0.7 %	Rh <sub>2</sub> (cDE)	10.1 %
Rh' Rh'' (CdE)	0 %	Rh <sub>1</sub> Rh <sub>2</sub> (CDE)	11.6 %

TABLE 2

Population	Number of determinations	Frequency of the types of Rh factor							
		Rh-	Rh <sub>1</sub>	Rh	Rh <sub>1</sub> Rh <sub>2</sub>	Rh <sub>0</sub>	Rh'	Rh''	Rh Rh''
New York { a b c	1 000	12.9	54.1	12.8	16.4	2.6	0.9	0.3	0
	818	13.5	55.6	15.8	12.0	1.7	1.1	0.3	0
	2,438	14.5	52.5	15.7	13.1	2.4	1.1	0.7	0.02
Negroes (New York)	223	8.1	20.2	22.4	5.4	41.2	2.7	0	0
Great Britain	927	14.8	54.9	12.2	13.7	2.5	0.7	1.3	0
Australia	350	14.9	54.0	12.6	16.5	0.6	0.9	0.5	0
Asiatic Indians	156	7.1	70.5	5.1	12.8	1.9	2.6	0	0
Chinese	132	1.5	60.6	3.0	34.1	0.8	0	0	0
Mexican Indians	98	0	48.0	9.2	41.8	1.0	0	0	0
Japanese { a b	150	1.3	37.4	13.3	47.3	0	0	0	0.7
	180	0.6	51.7	8.3	39.4	0	0	0	0
Filipinos	100	0	87.0	2.0	11.0	0	0	0	0
Indonesians	100	0	75.0	2	22	0	0	0	1
Present note Brazil (S Paulo)	138	15.2	55.2	10.1	11.6	5.8	1.4	0.7	0

The frequency of Rh types in São Paulo agrees with the North American, British, French<sup>11</sup> and Australian observations in white population, Rh negative (15.2 per cent) being slightly above the average because of the inclusion of some recognized Rh negative blood donors.

The type Rh<sub>1</sub> is by far the most frequent (55.2 per cent). The frequency for Rh<sub>0</sub> (5.8 per cent) is slightly higher, and for Rh<sub>2</sub> (10.1 per cent) is lower, than in the other statistics.

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## EDITORIAL

### FOLIC ACID, PERNICIOUS ANEMIA AND PENDULUMS

FOLIC acid, pteroyl glutamic acid (PGA), was heralded in 1946 as one of the "wonder drugs" of the year.<sup>1</sup> A few mg of the drug given orally was often found sufficient to cause a dramatic remission in typical cases of pernicious anemia. In such related conditions as sprue,<sup>2</sup> pernicious anemia of pregnancy,<sup>3</sup> "tropical" macrocytic anemia<sup>4</sup> and the like, the drug seemed to have an even greater therapeutic effect than did liver extract. The two chief questions which followed in the wake of the initial successes were, (1) how did it work so effectively even when given by mouth, and (2) would it prevent the development of neurologic manifestations?

The physiologic mechanisms by which PGA is effective are still quite obscure, but there can be no doubt that the various studies which have followed its introduction have illuminated certain aspects of the pathogenetic mechanisms in pernicious anemia.<sup>5</sup> Of greatest importance to the clinician however is whether or not PGA will in and of itself adequately protect the patient with pernicious anemia against the development of progressive involvement of the central nervous system, granted that other aspects of the deficiency state (bone-marrow, blood, gastrointestinal disturbances) are kept under control. During 1947 it became apparent that PGA often failed to prevent the development or progression of neurologic symptoms and that the signs of spinal cord involvement might develop explosively in patients taking the drug.<sup>6</sup>

Towards the end of 1947 an editorial entitled, "A Warning Regarding The Use of Folic Acid," appeared in the New England Journal of Medicine<sup>7</sup> which not only cast doubt on the ability of PGA to prevent neurologic involvement but even raised the possibility that the material might have a more or less directly harmful effect on central nervous system tissue. The editorial closed with the following statements: "sufficient evidence has accumulated to justify a warning that synthetic pteroyl glutamic acid (folic acid) should not be used in the treatment of pernicious anemia. In view of the reports of folic acid induced neurologic lesions in sprue this restriction should probably also apply to other nutritional macrocytic anemias. Consequently the use of folic acid as a therapeutic agent appears to offer *no new benefit but only risk to the patient*" (Italics ours.) So striking has been the impact of this editorial that many physicians have discontinued completely the use of PGA in their practice.

The above editorial stemmed in part from the work of Ross<sup>9</sup> and his collaborators, based on the treatment of 22 cases of pernicious anemia with folic acid.<sup>8</sup> In 7 cases, neurologic relapses developed, and in 4 there was progression of combined system degeneration. Neurologic relapse occurred with considerable suddenness and progressed rapidly in several patients, especially in those receiving large (10 to 25 mg) doses. This suggested the ingenious possibility that PGA, particularly when given in large doses, might contribute to dysfunction of the central nervous system by interfering with its metabolism of l(+) glutamic acid.

Some observations had already been made indicating that both the naturally occurring l(+) and d (-) glutamic acid are involved in nerve tissue metabolism (quoted by Ross<sup>9</sup>) Glutamic acid is one of the constituents of the folic acid molecule, and Ross points out that its position in the molecule suggests that it may enter into competition with the naturally occurring l(+) glutamic acid and thus interfere with normal nerve metabolism Ross implies that this interference might explain the greater frequency of neurologic relapse in patients receiving large doses of folic acid and the progression of neurologic disease in others

From the available evidence, it appears probable that PGA cannot be relied upon as the sole agent in the treatment of pernicious anemia since its use may not only prevent injury to the central nervous system but may actually be attended with harmful effects to nerve tissue Liver extract must therefore remain, at least for the present, the sheet anchor in the treatment of Addisonian pernicious anemia This does not exclude the possibility that PGA may *also* be useful (1) as an adjuvant to liver extract therapy and (2) as a more specific substance than liver extract in certain conditions related to Addisonian pernicious anemia but not identical with it

As regards the first possibility, Meyer,<sup>6</sup> for example, holds to the opinion that small doses of PGA, combined with liver extract injections, induce better remissions, both hematologic and neurologic, than either substance alone Ross et al.<sup>9</sup> also state 'These observations suggest that a combination of orally administered folic acid and parenterally injected liver extract may maintain a better hematologic status than either substance alone' In my own experience, small doses of folic acid, e g, 5 mg per day, have proved useful in the maintenance therapy of pernicious anemia, in conjunction with injections of liver extract at two to four week intervals This combination has seemed desirable on physiologic grounds since (1) an active therapeutic agent is given daily to supply a chronic deficiency state and (2) the patient receives at stated periods a deposit of a known and time tested material, i e, liver extract Under this regimen, all the patients treated have stated that their feeling of vitality is considerably improved, in addition, their red cell counts have tended to be higher than on liver extract alone No evidence of neurologic relapse has occurred As an adjuvant to liver extract therapy, PGA gives one the impression as being of distinct value

Has the drug a specific action in some cases which is not shared by liver extract? In 2 of our cases of neurologic pernicious anemia,<sup>10</sup> the administration of PGA was followed by a distinct neurologic remission, after prolonged liver extract therapy had proved ineffective In one of these cases, a reticulocytosis of 8.2 per cent developed at a red cell level of approximately 4.0 million per cu mm, whereas previously, liver extract therapy had resulted in only a 2.4 per cent response at a level of 3.8 million These findings seem to indicate that at least for these particular patients, folic acid was more nearly the required specific substance than was liver extract

In non-Addisonian pernicious anemia (that is, in conditions such as sprue, the macrocytic or pernicious anemia of pregnancy, the so-called tropical macrocytic anemia, and the megaloblastic macrocytic anemia of infancy) the efficacy of liver

extract, particularly of the refined type, leaves much to be desired. Lucy Wills,<sup>4</sup> for example, writes that "the liver principle is actively curative in pernicious anemia but does not seem to be the missing factor in nutritional macrocytic anemia." This "missing factor"<sup>11</sup> was alluded to by Watson and Castle as the 'Wills' factor' in an article dealing with the therapeutic inefficiency of parenteral liver extract in certain cases of atypical pernicious anemia. It is possible that the missing factor is PGA, especially since various workers have demonstrated that this material often results in a more marked therapeutic effect than does liver extract in cases of atypical pernicious anemia. Results in these cases suggest that the chemical acts specifically on certain enzyme systems that are not appreciably affected by liver extract. They also indicate that there may be different types of pernicious anemia brought about by varying mechanisms and even by different deficiencies.

The exact place of PGA in the armamentarium of therapy still remains to be clearly defined. That it is harmful to central nervous system tissue has not been conclusively demonstrated. Certainly the evidence at hand hardly justifies the categorical statements in the above mentioned editorial that the drug "should not be used in the treatment of pernicious anemia" and other nutritional macrocytic anemias and that its use appears to offer "only risk to the patient." These gloomy forebodings are hardly borne out by the results of many workers, particularly in the field of atypical pernicious anemia. Suarez,<sup>8</sup> for example, states that in the treatment of over 100 cases of sprue, not only has he seen no evidence of harm neurologically but the patients do better than with liver extract. Spies and co-workers<sup>12</sup> have recently shown that patients with nutritional macrocytic anemia can be maintained for as long as two years with folic acid as the sole medication without the development of subacute combined degeneration of the spinal cord.

Pendulums have a way of swinging too far in one or the other direction before they finally settle down. The 'PGA' pendulum, it would seem, has now swung far over on the reactionary side, as opposed to the enthusiastic left swing of 1946. This is perhaps only natural, in view of the disappointing results attending its use as the *sole* medication in typical pernicious anemia. However, when the exact nature of the liver extract factor has been defined, and when we know more about the specific enzyme systems concerned in the development of pernicious anemia, the pendulum should settle down, and PGA will then find its rightful place in the treatment of certain aspects of the pernicious anemia family of deficiency diseases.

WILLIAM DAMESHEK, M D

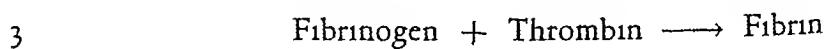
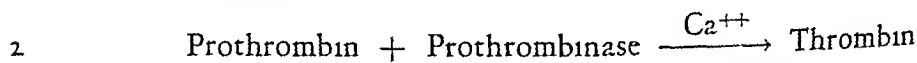
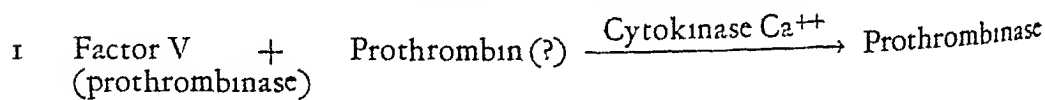
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## ERRATA

An error was made in Dr F H L Taylor's review of Dr Owren's book, "The Coagulation of the Blood, in the February issue of *Blood* (3 229, 1948) The formulae as printed were incomplete and should have read




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The editorial footnote in "Pernicious Anemia from Addison to Folic Acid," by Dr Russell L Haden, in the January *Blood* (3 24, 1948) should have read 'Wilkinson and Israels, Waldenstrom and others report that free hydrochloric acid occurs in approximately 1 case of 100' The term "achlorhydria" was used in error

# ABSTRACTS

JOSEPH L. ROSS, M.D., Editor

ABSTRACTS

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## HEMOGLOBIN, METHEMOGLOBIN AND BILIRUBIN METABOLISM

STERIC OXYGEN AFFINITY OF HEMOGLOBIN IN ELASMobrANCHS AND TURTLES *F. H. McCutcheon* From School of Veterinary Medicine, University of Pennsylvania. *J. Cell & Comp. Physiol.* 29: 333-344, 1947

Knowledge that fetal goat hemoglobin has a higher affinity for oxygen than maternal hemoglobin has led to further investigations along developmental and comparative lines. In the present study, various elasmobranchs and turtles were obtained in the adult, fetal and embryonic forms. In general, sharks had a higher loading capacity and a lower unloading capacity for oxygen than the rays. Unlike other animals, the fetal ray had an affinity for oxygen like that of the adult. Lack of material did not permit a study of the more embryonic stages. In the turtles, the terrestrial forms had a higher affinity than the aquatic forms. In the development of turtles, no changes in oxygen affinity occur until after the embryos hatch. By the end of the second year the adult type of hemoglobin is established. These and similar results may be correlated with ontogenetic, phylogenetic, ecologic and physiologic factors.

O P J

CYANOSIS IN INFANTS IN RURAL AREAS (WELL-WATER METHAEMOGLOBINAEMIA) *H. Medow, W. C. Guest, and M. Victor* From the Department of Pediatrics, University of Manitoba and the Department of Medicine, Winnipeg General Hospital, Winnipeg, Manitoba, Canada. *M. A. J.* 56: 505-508, 1947

One certain and one probable case of methemoglobinemia are reported in infants whose formulae included well water containing from 110 to 250 parts of nitrate per million. The upper limit of safe nitrate content is given as 10 parts per million. In both cases cyanosis disappeared without treatment within one or two days after the use of well water was discontinued.

Four similar reports, including one fatal case, in the literature are cited, all cases occurring in infants during the first two months of life. It is suggested that the determining factors are body weight, nitrate content of well water and amount of water ingested. The incidence of well-water methemoglobinemia would, therefore, be highest in areas where farm sanitation is poor, wells poorly constructed and where dried milk mixtures are extensively used.

The efficacy of methylene blue and ascorbic acid in treating methemoglobinemia is briefly discussed. Prompt recognition of the condition is most important, since spontaneous recovery follows when use of contaminated well water is abandoned, provided the methemoglobin content of the blood has not risen too high.

L E Y

THE ROLE OF PYRIDINE NUCLEOTIDES IN THE REDUCTION OF METHEMOGLOBIN *H. R. Gutmann, B. J. Jandorf, and O. Bodansky* From the Biochemistry Section, Medical Division, Army Chemical Center, Edgewood Arsenal, Maryland. *J. Biol. Chem.* 169: 145-152, 1947

The early studies of Warburg on the mechanism of erythrocyte methemoglobin reconversion are extended by these authors. Methylene blue was used as a part of the system because of its known ability to accelerate the reaction. While glucose functions as a substrate in the intact erythrocyte, hexose diphos-

phate or lactate are necessary in hemolysates. Methemoglobin reversion occurred in the hemolysate when nicotinamide was employed to suppress pyridine nucleotide hydrolysis. The essential substrates for the reduction of methemoglobin in these experiments appeared to be reduced DPN and phosphorylated glucose or lactate. These observations, unfortunately, do not throw any light on the one clinical state in which the cell reconversion mechanism does not function—congenital methemoglobinemia. This would suggest that the reconversion system is more complex than herein described.

C. A. F.

**SIGNIFICATION BIOLOGIQUE DES CORPUSCULES DE HEINZ. LEUR RAPPORT AVEC LES VERDOGLOBINES** (Biologic significance of the Heinz corpuscles, their relations with the verdoglobins) *A. Gajdos and G. Tipter*. Travail de la clinique médicale de l'Hôtel-Dieu (Paris). *Sang* 18: 35-43, 1947.

In 1890, Heinz was the first to notice the appearance of basophilic corpuscles in the red cells during phenylhydrazin intoxication. These corpuscles are stained by supravital staining, but in contrast to reticulocyte granulations, they disappear when later stained by a panoptic method (May-Grünwald Giemsa). Several hemolytic poisons, and some sulfonamide drugs bring on the corpuscles, in vivo as well as in vitro. Their chemical nature and their biologic significance have been the subjects of numerous researches. Some think they are due to methemoglobin, others that they are related to verdoglobins (intermediary substances between hemoglobin and bile pigments).

The experiments of A. Gajdos and G. Tipter bring new arguments for the close relations between Heinz corpuscles and verdoglobins. They show that all substances able to transform hemoglobin into verdoglobin induce Heinz corpuscles in vivo and in vitro, in particular ascorbic acid. Moreover, the same factors are acting (pH, temperature, speed of formation) for both verdoglobin and Heinz corpuscles. Thus, these corpuscles seem to represent an alteration of the hemoglobin in the red cells by hemolytic poisons.

J. P. S.

**ICTERUS NEONATORUM. ITS INCIDENCE AND CAUSE** *L. Findlay, G. Higgins, and M. W. Stanier*. From the Department of Biochemistry, Radcliffe Infirmary, Oxford, England. *Arch. Dis. Childhood* 22: 65-74, 1947.

The authors studied the incidence of physiologic icterus in the newborn and attempted to decide whether it is due chiefly to hemolysis or to some other factor, such as hypofunction of the liver. They found first that up to 81 per cent of newborn infants had a plasma bilirubin in excess of 1 mg per cent (i.e., laboratory icterus), although much fewer (e.g., 18 per cent of one group) had clinical jaundice. At two weeks of age, all infants studied had levels of 1 mg per cent or less. The level of neonatal plasma bilirubin roughly paralleled that of the cord blood, but there was not a strict correlation, nor was there a correlation between the plasma bilirubin and the maturity of the fetus. Jaundice, however, was more likely to occur the more premature the baby.

pregnancy	jaundice
32-35 weeks	100% of subjects
36-37	65%
38-39	47%
40 (term)	47%

It is generally taught that physiologic icterus is the result of hemolysis, which is said to cause the fall in hemoglobin and red cell count from the prenatal to neonatal levels. Against this concept the authors adduce the following data:

1. The most rapid fall in hemoglobin and red count occurs in the second week of life, whereas the hyperbilirubinemia occurs largely in the first week. The fall in blood values continues for months, long after the jaundice is gone. (*L. Findlay Arch. Dis. Childhood* 21: 195, 1946.)
2. The rate of fall of hemoglobin was found to be the same in jaundiced and in nonjaundiced infants.
3. None of the usual concomitants of hemolysis (reticulocytosis, normoblastosis) occurred postnatally, whereas fetal umbilical blood was found regularly to contain increased reticulocytes and normoblasts.
4. The erythrocytes of fetal blood showed increased fragility to hypotonic solutions, those of neonatal blood showed decreased fragility. (*Arch. Dis. Childhood* 20: 64, 1945.)

The authors believe that neonatal physiological icterus is the result of immaturity of function of the liver which like many other organs is relatively immature at birth. In utero, they postulate, the fetus's bilirubin is excreted largely by way of the maternal placenta, so that not till birth does the liver begin to excrete bilirubin. Attempts to study neonatal liver function gave inconclusive results, but more bilirubin was found in the feces of nonjaundiced than of jaundiced infants.

S E

## HEMOGLOBINEMIA AND HEMOGLOBINURIA

A NOTION STUDIES OF HEMOLYSIS PAROXYSMAL (COLD) HEMOGLOBINURIA. *P. F. Wagle, W. H. Zinkman and L. A. Sellers*. From the Department of Medicine, Johns Hopkins University, Baltimore, Maryland. *Am J Med* 34: 346, 1947.

A preliminary report is made of studies carried out on the blood of a syphilitic patient with paroxysmal cold hemoglobinuria. It was shown that saturation of the patient's blood with carbon dioxide produced hemolysis of the erythrocytes, an effect which was not dependent on a reduction of the pH of the serum (as is the case in paroxysmal nocturnal hemoglobinuria). This hemolysis was dependent on the presence of a hemolysin in the patient's serum.

Inhibition of the carbonic anhydrase of the erythrocytes with cyanide or with sulfanilamide blocked the hemolytic action of chilling. This blockage was due to inhibition and not to destruction of the carbonic anhydrase.

Microscopic observation of erythrocytes being hemolyzed by the patient's serum showed that these cells underwent considerable swelling and morphologic changes just prior to lysis.

J F R

RAYNAUD'S PHENOMENON, WITH PAROXYSMAL HEMOGLOBINURIA, CAUSED BY COLD HAEMAGGLUTINATION. *C. H. Whittle, A. Lyell, and M. Getman*. *Proc Roy Soc Med* 40: 500-502, 1947.

The chief point of interest in this patient with a five year story of anemia and a two year story of episodes of blueness, tingling, and numbness of the hands on exposure to the cold, is the extraordinarily high titer of the autoagglutinin circulating in the blood serum. The patient had no physical abnormalities except after chilling, when bluish red blotches appeared over the fingers, hands, and tips of nose and ears. The blood studies showed a persistent macrocytic anemia with reticulocytosis and thrombocytosis, and normoblastic hyperplasia of the bone marrow. The titer of autoagglutinin was 1:2,000,000 at ice-box temperature, and the red cells were very fragile to mechanical trauma when kept at low temperatures. Actual rupture of the red cells was visible *in vivo* under the capillary microscope.

S E

MARCH HEMOGLOBINURIA. *A. M. Bell*. From Alvinston, Ontario. *Canad M A J* 57: 43-46, 1947.

The case reported is that of an apparently normal 18 year old soldier who for three months exhibited hemoglobinemia, hemoglobinuria, albuminuria and abdominal distress after walking for periods as short as thirty minutes. For at least part of the three months the appearance of symptoms seemed to depend upon the presence of food in the upper digestive tract. Further study of this phenomenon was prevented by spontaneous remission. A brief review of march and other types of paroxysmal hemoglobinuria is given and the association of albuminuria with hemoglobinuria is discussed.

L E Y

PREPARATION OF HEMOGLOBIN SOLUTIONS FOR INTRAVENOUS INFUSION. *P. B. Hamilton, L. E. Farr, A. Hiller and D. D. Van Slyke*. From the U. S. Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research, New York. *J. Exper. Med.* 86: 455-463, 1947.

A method is described for preparation of sterile, nonpyrogenic solutions of oxyhemoglobin which have the approximate protein content and electrolyte composition of plasma. The procedure involves precipitation of stroma by adding 0.1 N HCl to laked red cells and removal of excess potassium by treatment with sodium zeolite.

The authors report that large volumes of solution can be rapidly prepared with 95 to 98 per cent of the hemoglobin in active form capable of combining with oxygen. Solutions stored at 4 C. showed no



conversion of hemoglobin to methemoglobin over a period of two and a half months, but a small and variable conversion was detected over a six month period

L E Y

PREPARATION OF DRIED HEMOGLOBIN WITHOUT LOSS OF ACTIVITY *L E Farr, A Heller and D D Van Slyke*  
From the U S Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research,  
New York J Exper Med 86 465-475, 1947

The methods used for freezing, drying and preserving plasma in vacuo were successfully applied to hemoglobin solutions, but only after hemoglobin was deoxygenated to prevent formation of methemoglobin during the drying process. It was found that deoxygenated hemoglobin dried and preserved in vacuo retained all its oxygen-binding capacity for 180 days when stored at temperatures from 4 to 30 C., for 92 days at 38 C and for 7 days at 56 C. Dried deoxygenated hemoglobin was partly converted to methemoglobin by even momentary contact with oxygen, but it was stable when dissolved in water before being exposed to air and could be stored for months at 4 C, in contact with air, without significant loss of activity. The dried hemoglobin had a foam structure which caused it to dissolve immediately upon contact with water.

Deoxygenation was accomplished by repeated shaking of hemoglobin solutions under diminished pressure. All but traces of oxygen were removed by alternately de-gassing the solutions and saturating them with oxygen-free nitrogen. It is pointed out, however, that after completion of this work, Pennell, Smith and Werkheiser reported a procedure for deoxygenation by action of enzymes in laked cells after addition of nicotinic acid and glucose. This method is considered better adapted to large scale preparations.

L E Y

RENAL EFFECTS OF HEMOGLOBIN INFUSIONS IN DOGS IN HEMORRHAGIC SHOCK *P B Hamilton, A Heller and D D Van Slyke*  
From the U S Navy Research Unit at the Hospital of The Rockefeller Institute  
for Medical Research, New York J Exper Med 86 477-487, 1947

Dogs were bled, 50 cc per kilo body weight, and the blood withdrawn was quickly replaced by equal volumes of 0.9 per cent NaCl solution, heparinized dog plasma, or 7 per cent oxyhemoglobin or methemoglobin solution. The effects of more prolonged hemorrhagic shock were not studied. When the blood was not replaced, oliguria or anuria developed and the urea clearance was depressed for several hours after bleeding, but renal function returned to normal within twenty-four hours. Infusion of 0.9 per cent NaCl solution or plasma promptly relieved the oliguria and elevated the urea clearance. Injection of oxyhemoglobin solution also relieved oliguria promptly, but in some cases there followed a period of three to five days in which urea clearance was depressed to about 25 per cent of normal and plasma urea nitrogen was moderately elevated. The clearance then returned to normal during the next five or six days. No histologic observations are reported.

When acidosis was produced before bleeding by giving  $\text{NH}_4\text{Cl}$ , the results after infusion of plasma or hemoglobin were unchanged, but since hemorrhage itself caused nearly as much acidosis as the  $\text{NH}_4\text{Cl}$ , acidosis was not excluded as a factor in these experiments.

It was shown that methemoglobin was rapidly converted into active hemoglobin after injection, and that there was no significant difference between infused oxyhemoglobin and methemoglobin either in effect on renal function or in rates of disappearance from circulation and excretion in the urine.

The authors conclude that, although the immediate effects of hemoglobin solutions are favorable in treating posthemorrhagic shock in dogs, the subsequent transitory depression of urea clearance indicates sufficient possibility of renal damage to prevent recommending the use of such solutions as blood substitutes.

L E Y

RENAL DAMAGE FOLLOWING INTRAVASCULAR HEMOLYSIS *E L Burwell, T D Kinney, and C A Finch*  
From the Department of Medicine and Pathology, Peter Bent Brigham Hospital and Department of Pathology, Harvard Medical School, Boston, Massachusetts New England J Med 237 657-665, 1947

A case of massive intravascular hemolysis following a therapeutic abortion is presented and the management of the subsequent anuria is discussed in detail. The etiology of the hemolysis was attributed to

either more self-therapeutically or indirectly by way of the uterus or by peristalsis reaction to an orally injected drug. Dr. Hetschline has called the attention of the author to the article by Hill on Post-abortual and Puerperal Gas Gangrene (J. Clin. & Exptl. Surg. 1948) and the similarity of this case to the reported cases of hemolysis due to *Brucella abortus*.

The recovery of the fetal leucocytes showed by renal function test 2 days after the death of the patient three months later with acute hepatitis. The renal atrophy in leucocytes as found to consist of only minimal scarring. The histologic picture of the pathogenesis of the morphologic response and its treatment are reviewed.

C A I

THE PATHOGENESIS OF THE RENAL INJURY PRODUCED BY THE POORLY HEMOLYSED OR METHEMOGLOBINIC Hb. H. H. Hume, H. B. Burt, N. K. Johnson, and H. A. Zerk. From the Department of Pathology, Yale University School of Medicine, New Haven, Conn. J. Exp. Med. 63: 353-356, 1947.

Severe and persistent impairment of renal function was produced in dogs by extreme intravascular hemolysis due to the repeated intravenous injection of large amounts of dog hemoglobin and methemoglobin. Methemoglobin caused the experimental injury in an equal amount of oxhemoglobin. In oliguric dogs, anoxemia and reduction of creatinine clearance followed injection of methemoglobin in amounts resulting in plasma concentrations of 1 Gm. per 100 cc., while much higher concentrations were tolerated by dogs with greater urine output. Acidosis produced by administration of 0.1 N hydrochloric acid appeared to have less effect than oliguria in the production of renal damage after injections of methemoglobin. In acidotic dogs, the pH of the urine typically complied with the onset of hemoglobinuria, sometimes from approximately 7.2 to 7.0, but no explanation of this change is given.

Kidneys of the experimental animals were examined by the ferrocyanide histochemical technique to determine whether the renal tubules were functionally obstructed. In addition, casts were teased out of frozen sections, dissolved in buffer solutions and the absorption spectra and solubilities of their pigments were determined.

Material filling the lumina of the tubules was found to be chiefly methemoglobin in concentrated solution of gel-like consistency. No evidence of formation of pigments such as hemochromogen or hematin, insoluble at the pH of the urine, was found. Obstruction to the flow of urine through the tubules appeared to be an important factor in early reduction of renal function and was attributed to increased viscosity of tubular contents. It is worthy of note, however, that the concentrations of hemoglobin and methemoglobin produced in the plasma in these experiments were for the most part considerably in excess of those encountered clinically. The mechanism of cast formation under these conditions is not yet clear.

Necrosis of proximal convoluted tubule cells was found as a late lesion and was deemed a contributing factor in the persistent depression of renal function. Following disappearance of most of the intratubular pigment, many collapsed tubules lined with hemosiderin-filled cells were found and were considered to represent nonfunctioning nephrons.

Direct measurements with a T-tube cannula inserted into the renal vein in two animals failed to reveal any reduction of renal blood flow following injection of methemoglobin in amounts sufficient to produce renal injury. It should be pointed out, however, that the method here employed might fail to measure a reduction in effective renal blood flow, if such occurred, due to diversion of flow from cortex to medulla by recently discovered shunting mechanisms.

L E Y

## IRON METABOLISM

THE PATHOGENESIS OF CYTOSIDEROSIS (HEMOCHROMATOSIS) AS EVIDENCED IN MALNOURISHED AFRICANS. J. Gillman and T. Gillman. From the Department of Anatomy, Medical School, University of the Witwatersrand, Johannesburg, South Africa. Gastroenterology 8: 19-23, 1947.

Based on numerous histochemical studies of liver biopsy material and tissues obtained postmortem from nutritionally deficient African natives, many of whom presented clinical and pathologic evidences of hemochromatosis, a theory is proposed by the authors regarding the sequence of events operative in the pathogenesis of that disease. Initially, as a complication of malnutrition, commonly pellagra, there occurs a degradation of intracellular iron compounds, possibly cytochrome or catalase. During this stage of the process a striking porphyrin-like fluorescence is demonstrable on examination of liver tissue, it is

presumed that other tissues are likewise involved in this process of deterioration. Iron liberated with the liver is excreted into the bile, then promptly and completely reabsorbed into the intestinal mucosa. The cells lining the intestinal tract, similarly affected by the underlying metabolic disorder, become laden with iron. Iron accumulates in the lymphocytes of the tunica propria and subsequently enters the lymphatic circulation, chains of mesenteric lymph nodes becoming progressively pigmented. Eventually sufficient iron becomes absorbed into and distributed by the systemic circulation to effect a generalized siderosis of the entire reticulo-endothelial system, including the Kupffer cells, which now serve as an additional source of iron delivered to the upper abdominal lymph glands, already pigmented from iron-containing lymph derived from the liver itself. In severe cases these glands draining the small intestine, stomach, pancreas and liver become extensively involved. Successful arrest of the process necessarily involves an attack on the fundamental disorder of intracellular metabolism, which is believed to be secondary to a nutritional deficiency.

C.P.E.

**HAEMOSIDEROSIS PULMONUM.** *P. Haussan* From the Medical Department of the City Hospital of Bergen, Bergen, Norway. *Acta Paediatr.* 34: 103-111, 1947.

A case of this unusual syndrome was diagnosed clinically from the appearance of the pulmonary roentgen picture and the hematologic status. This was characterized by the mixture of sideropenic and hemolytic symptoms that is typical for the malady. The hemoglobin responded well to iron therapy. There was noted a constant reticulocytosis, urobilinuria and periodically increased serum bilirubin. Of special interest is the demonstration of large amounts of hemosiderin in microscopic sections of the patient's sputum. Another finding that may possibly be of importance for the understanding of the disease in the future was a sarcoid-like structure found in an excised cervical lymph gland. No autopsy was performed but the clinical diagnosis was quite convincing. It is probable that this syndrome is sometimes overlooked and should be diagnosed more frequently.

J.W.

**STUDIES ON FREE ERYTHROCYTE PROTOPORPHYRIN, PLASMA COPPER, AND PLASMA IRON IN NORMAL AND IN PYRIDOXINE-DEFICIENT SWINE.** *G. E. Cartwright and M. M. Wintrobe* From the Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah. *J. Biol. Chem.* 172: 557-565, 1948.

This is an extension of previous studies by these authors on the anemia produced by pyridoxine deficiency in swine. Normal values of erythrocyte protoporphyrin in swine were found to be  $118 \pm 43 \gamma$  per 100 cc, serum copper  $206 \pm 26.3 \gamma$  per cent, and serum iron  $169 \pm 38.8 \gamma$  per cent. In pyridoxine deficiency, protoporphyrin was reduced to an average of  $47 \pm 13.6 \gamma$ . Copper was reduced to  $160 \pm 38.8 \gamma$  per cent while serum iron showed an increase to  $408 \pm 166.6 \gamma$  per cent. Urinary coproporphyrin was not altered. Following intravenous administration of pyridoxal and pyridoxamine, serum iron dropped to normal limits within twenty-four hours, and erythrocyte protoporphyrin rose over several days to about normal limits.

The authors suggest that in view of the reduced free erythrocyte protoporphyrin, the fundamental disturbance in erythropoiesis may be a failure to synthesize protoporphyrin. The elevation of serum iron and tissue hemosiderosis is a natural sequence of this block in hemesynthesis.

These investigations are the first to suggest such a defect in protoporphyrin metabolism as a cause of anemia. Investigations along this line are warranted in Cooley's anemia which has certain similarities to the anemia of pyridoxine deficient swine.

C.A.F.

**REDUCTION OF IRON BY FOODS IN ARTIFICIAL GASTRIC DIGESTION.** *E. R. Kirch, O. Bergheim, J. Kleinberg, and S. James* From the Department of Biological Chemistry, College of Medicine, and the Department of Chemistry, College of Pharmacy, University of Illinois, Chicago, Illinois. *J. Biol. Chem.* 171: 687-694, 1947.

A method is described of measuring ferrous and ferric iron in the presence of each other. Under conditions simulating gastric digestion, the degree of reduction and binding by various foods and biologic materials was tested. The reduction of iron varied with different foods from 0 to 98 per cent. Ascorbic acid and proteins were felt to be in part responsible for this reduction.

Recent studies have indicated that dietary factors are important in determining iron absorption. Since there is some evidence that iron must be in the ferrous form for absorption to occur, this reduction capacity of the diet may be a determining factor

C A F

VARIATIONS DE LA SIDEREMIE ET DE LA CUPREMIE AU COURS DE LA REGENERATION DES ANEMIES PAR LA METHIONINE (Variations in the Blood Iron and Copper Concentrations Found in the Course of Anemias Treated with Methionine) *A Lafontaine and A Gajdos* *Sang* 18 242-246, 1947

The authors describe 4 cases of human anemia treated with 2 Gm methionine daily, in which the amount of serum iron and copper were estimated (Iron estimated by the method of Heilmeyer and Plotner, modified by Lederer and De Mouschalt, copper estimated by the method of Callan and Henderson, modified by Briskas) In all 4 cases, the amounts of serum iron and copper increased considerably at the beginning of the treatment, despite a diet poor in these elements. The increase of serum iron is more important than that of serum copper. While the anemia regresses, the copper, and above all the serum iron, decreases. It seems, therefore, that methionine aids the mobilization of iron and copper. It would be interesting to give iron with methionine therapeutically.

J P S

## LEUCOPENIA AND AGRANULOCYTOSIS

THE EFFECT OF TRIMETHYLOAZOLIDINE DIONE (TRIDIONE) ON THE BLOOD *J P Davis and W G Lennox*  
From the Department of Neurology, Harvard Medical School and The Children's and Infants' Hospital, Boston, Mass *J Pediat* 31 24-33, 1947

Periodic hematologic examinations of 127 patients receiving tridione demonstrated, as the only alteration of possible serious significance, the gradual development of a definite neutropenia in 63 per cent, the neutrophil counts in this group falling to values between 600 and 1600 per cubic millimeter. An additional 7 per cent exhibited a mild neutrophilic depression, counts descending below 2500 per cubic millimeter. The total leukocyte count was relatively unaffected, due to a concomitant absolute lymphocytosis. The occurrence of granulopenia was unrelated to dosage of the drug, or to the therapeutic schedule employed, no premonitory or accompanying clinical manifestations or hematologic abnormalities were observed, in no instance was its onset abrupt, or its progression rapid. Restoration of a normal granulocyte count promptly and invariably followed discontinuance of the drug. Therapy was resumed in 3 cases without recurrence of neutropenia. It is emphasized that, whereas drugs with this type of molecular structure may be potential bone marrow depressants, the administration of tridione may be safely controlled and the development of a marked or irreversible neutropenia forestalled by conducting monthly hematologic examinations which should always include a leukocyte differential, as well as total white cell count.

C P E

AGRANULOCYTOSIS AND HEPATOCELLULAR JAUNDICE TOXIC REACTIONS FOLLOWING PROPYLTHIOURACIL THERAPY *H J Livingston and S F Livingston* Brooklyn, N Y *J A M A* 135 422-425, 1947

The 68 year old hyperthyroid patient who forms the subject of this report developed sudden acute neutropenia (agranulocytosis) after the previously uneventful administration of some 13,400 mg of propylthiouracil in a period of ten weeks. The blood showed 1,000 white cells with no granulocytes at all, and granulocytes were virtually absent from the marrow puncture. About the same time, however, a non-hemolytic jaundice supervened, attributed to hepatocellular damage caused by the propylthiouracil. Treatment consisted of penicillin, streptomycin, amino acids, liver extract, vitamin injections, and blood transfusion, and improvement gradually occurred. The thyrotoxicosis remained unchanged and required subsequent thyroidectomy.

It is impossible to evaluate this curious case from the published article, in which no tables were included because of lack of space. The neutropenia is amenable to explanation on the basis of known damage to granulocytopoiesis in the marrow, which has been well demonstrated in the case of various drugs, including thiouracil, and has been reported for half a dozen cases with propylthiouracil. The occurrence of hepatic involvement must be very rare or even unique, and the question of its etiology remains unexplained from the data presented.

S E

LEUKOPENIA FOLLOWING THE USE OF PROPYLTHIOURACIL *W J Eisenrenger and J M Steele* From the New York University Division of the Lenox Hill Hospital, New York *J A M A* 135 510-11, 1947

The authors report a patient of 65 with hyperthyroidism, who developed a leukopenia and neutropenia (white cells 3,400, neutrophils, 40 per cent) after administration of some 36 grams of thiouracil in a period of five months. Cessation of drug therapy was followed by prompt return of the leukocyte and neutrophil values to normal, but relapse in the hyperthyroidism occurred within two months. The patient was therefore put on propylthiouracil, 50 mg three times a day, and later 75 mg daily. There was no effect on the blood counts, but the thyrotoxicosis remained uncontrolled, and the dose of propylthiouracil was raised to 200 mg daily. This increase in dosage was followed by a rapid fall of white count to 2,100 per cu mm, with 22 per cent neutrophils. This drug, too, was therefore stopped, and the count returned to normal values. Thyroidectomy was subsequently used to control the hyperthyroidism.

According to the authors, of some 471 cases treated with the drug this is the seventh case in the literature of leukopenia due to propylthiouracil.

S E

## THE R<sub>H</sub> FACTOR AND ERYTHROBLASTOSIS FOETALIS

ERYTHROBLASTOSIS FOETALIS OR HAEMOLYTIC DISEASE OF THE NEWBORN *L K Diamond* From the Children's and Infants' Hospitals and the Harvard Medical School, Boston, Mass. *Proc Roy Soc Med* 40 546-550, 1947

This is the substance of a lecture delivered by Diamond in England in May 1947. Several points covered in this review are of interest.

1. About 13 per cent of all marriages involve an Rh negative woman and an Rh positive man, but only one in 150 of all deliveries results in an infant with erythroblastosis foetalis. In other words, less than one susceptible woman in 20 is sensitized by pregnancy alone, whereas a single transfusion of Rh positive blood, followed by subsequent pregnancy, in an Rh negative woman, increases the chances that the child will be erythroblastotic from 5 per cent to over 50 per cent.

2. The results of treatment of affected infants over a period of some twenty years are reviewed. During fifteen years in which the only treatment was transfusion of compatible blood, usually from the baby's father (i.e., Rh positive), the mortality, including stillbirths, was 40 per cent. During three years in which treatment consisted of multiple small transfusions with Rh negative blood, the mortality was 30 per cent. In the period of 1944 to 1946, when early delivery in affected cases was combined with Rh negative transfusions after delivery, the mortality was 20 per cent. The newest technique, combining early delivery with careful studies at birth and, when indicated, exsanguination-transfusion of affected infants, resulted in a mortality of about 10 per cent in the first 50 cases. Of those that recovered, most responded to the single procedure of exsanguination-transfusion, and only a few needed a subsequent small transfusion in the third week of life.

3. Diamond gives flexible indications for exsanguination-transfusion. The procedure is indicated (a) when the mother has Rh antibodies in the serum, and the infant has clinical erythroblastosis foetalis, (b) when the mother has Rh antibodies in the serum, the infant has no symptoms at birth, but cord blood shows the baby to be Rh positive and his serum to have Rh antibodies. If the infant looks good and, although Rh positive, has no free antibody by suitable tests, no therapy is attempted at birth, in most of this group, no treatment became necessary, although a few such infants subsequently required a single transfusion of blood.

The experiences of Diamond are in keeping with generally held ideas concerning the mechanisms of erythroblastosis foetalis.

S E.

HAEMOLYTIC DISEASE OF THE NEWBORN IN SOUTH AFRICAN NATIVES. REPORT OF THREE CASES *A Zentgraf* From the South African Institute for Medical Research, Johannesburg. *South African M J* 21 794-796, 1947

The 3 cases of erythroblastosis foetalis cited in this report are presumably the first to be described among South African natives. It is emphasized that only 5 per cent of the natives are Rh negative and that only 18 per cent of Bantu bloods react with anti-C (anti-Rh<sup>1</sup>) serum as compared with 70 per cent among Europeans.

peans It is extremely rare, moreover, to encounter native bloods which react with anti-E (anti-Rh'') serum (30 per cent among Europeans)

All of the 3 cases reported occurred in infants born of Rh positive mothers, and in each case incomplete antibodies were present while abnormal agglutinins were absent In the first case the baby's type was CDE (Rh<sub>1</sub>Rh<sub>+</sub>) and the mother's type was CDe (Rh<sub>1</sub>), incomplete antibodies against CDE (Rh'') were found in the maternal serum—a finding of rarity even among Europeans In the second case the baby's type was CDe (Rh<sub>1</sub>), the mother's Cde (Rh'), anti-D (Rh<sub>0</sub>) incomplete antibodies were present In the third case the baby's type was CDe (Rh<sub>1</sub>), the mother's CDe (Rh<sub>0</sub>), and incomplete anti-C (Rh') antibodies were detected

L E Y

THE INTELLIGENCE QUOTIENT IN CASES OF RHEUS INCOMPATIBILITY *M Greck Arch Dis Childhood* 22 181-182, 1947

Forty-three unselected cases of erythroblastosis fetalis who survived were studied for intellectual development by means of Gesell's development quotient (DQ) and standard intelligent quotients (IQ) It was found that 5 children who had gross neurologic involvement consistent with kernicterus, all had low DQ and low IQ In all, jaundice and anemia had been present after birth Only two other children had slightly low DQ and IQ On the other hand, 7 other children with slight physical abnormalities not suggestive of kernicterus, had normal DQ and IQ, and in 2 of these, jaundice had been extremely severe

These results suggested that severe mental defects in such patients is closely associated with the physical characteristics of neurologic damage In the absence of such damage due to kernicterus, the mentality is perfectly normal Erythroblastosis fetalis in itself, in other words, has no particular relationship to impaired intellectual development, unless the latter is due directly to brain damage attributable to kernicterus

S.E

DETERMINATION OF ANTI-RH ANTIBODY IN INFANTS WITH ERYTHROBLASTOSIS FETALIS *W E Wheeler and M L L Scholl From the Department of Pediatrics, Ohio State University College of Medicine, and the Children's Hospital, Columbus, Ohio Am J Dis Child* 74 274-282, 1947

A superior method for liberation and demonstration of both complete and incomplete anti-Rh antibodies attached to erythrocytes of babies with erythroblastosis is described Washed packed red cells from the infant are divided into two equal portions, one of which is resuspended in 3 volumes of saline, the other in a similar amount of a 20 per cent solution of bovine albumin Both portions are heated at 45 to 50 C for 30 to 40 minutes, then centrifuged rapidly at the same temperature and the supernatant fluids tested with Rh<sub>1</sub>, Rh<sub>2</sub> and Rh negative cells of the same OAB group In 10 of 11 cases, the albumin supernate gave a positive conglutination test, but all of the saline supernates failed to produce agglutination In 6 cases, however, Rh positive cells incubated in the saline supernates became sensitized as demonstrated by their agglutination when anti-human-serum rabbit serum was added

It is emphasized that these procedures establish the specificity of the antibody attached to the baby's cells, whereas the Coombs, Mourant and Race (CMR) test with rabbit serum, when carried out by itself, merely demonstrates the presence or absence of cell-bound globulin The ease with which Rh antibodies are separated from cells by heat suggests to the authors that the antibodies have low avidity They offer this as an explanation for the puzzling situation present at birth in which there may be found free antigen in agglutinable red cells, free antibody in the baby's serum and antibody combined with antigen in the red cells It is further suggested that after an Rh positive cell is damaged, Rh antibody may be released *in vivo* to attack fresh Rh positive cells introduced into the circulation, and that antibody may not be bound to fresh cells permanently The importance of these considerations in exchange transfusions and in the transfusion of Rh positive cells to erythroblastotic infants is stressed

L E Y

STUDIES ON THE CONGLUTINATION REACTION, WITH SPECIAL REFERENCE TO THE NATURE OF CONGLUTININ *A S Wiener, J G Hurst, and E B Sonn-Gordon From the Serological Laboratory of the Office of the Chief Medical Examiner of the City of New York J Exper Med* 86 267-284, 1947

The nature of conglutinin was explored by adding plasma and plasma constituents singly and in combination to Rh<sub>1</sub> and Rh<sub>2</sub> cells sensitized in respective tubes with serial dilutions of Rh antisera containing incomplete antibodies. Supernatant fluid was removed as completely as possible from the cells before conglutinin was added.

It was found that dilution of oxalated human plasma with more than an equal volume of saline destroys its ability to produce conglutination of cells sensitized by univalent antibody. Plasma showed greater conglutinating activity than serum, presumably due to the presence of fibrinogen, which the authors consider an important component of the colloidal complex of proteins making up conglutinin. Heating at 56 C. for 30 minutes weakened slightly the activity of plasma by causing precipitation of fibrinogen, but similar treatment of serum improved its activity slightly.

Although there was little variation in conglutinin activity of sera from different normal adults, fetal plasma and serum yielded much lower titers. The rapid increase in conglutinin content of the blood after birth is correlated with the abrupt onset of icterus gravis. The use of whole citrated blood in exchange transfusion of an erythroblastotic baby caused a rise in total plasma proteins and conglutinating activity, whereas replacement of transfused plasma with saline eliminated this unfavorable change in the infant's plasma.

Although 25 per cent human albumin solution yielded titers only half as high as plasma, a mixture of 1 part albumin with 3 parts plasma produced a fourfold increase in titer of the plasma. Addition of more or less than this optimal amount of albumin resulted in lower titers. Albumin solutions of less than 12.5 per cent and immune globulin solutions of less than 4.6 per cent concentration had little conglutinin activity, but mixtures of these dilute solutions in optimal proportions showed activity greater than that of plasma. On the basis of these observations it is emphasized that intensity of conglutination does not depend merely on the total protein content of the medium of suspension. It is further suggested that there may be substances in normal plasma which tend to maintain albumin and globulin in molecular dispersion, and that fractionation may render albumin and globulin less hydrophilic, thus increasing their tendency to form colloidal aggregates.

LEY

## MALIGNANT LYMPHOMA

LA DIAGNOSI CITOLOGICA DEL GRANULOMA MALIGNO PER PUNTURA MIDOLLARE, SPLENICA E GHIANDOLARE (Cytologic diagnosis of Hodgkin's disease by bone marrow, splenic and lymph node aspiration) L. Battistoni and F. Perazzini. From the Istituto di clinica medica generale e terapia medica della università di Bologna. *Hematologica* 30: 89-112, 1947.

The authors studied 7 cases of Hodgkin's disease by means of organ punctures and biopsies. They found in bone marrow punctures, an increase in plasma cells which were frequently atypical, but only occasionally noted Sternberg cells. In the splenic punctures there was an inversion of the normal ratio lymphocytes/granulocytes as a result of increase in the granulocytes, and Sternberg cells were often noted. In lymph node punctures, Sternberg cells were found in all cases and there was a striking cellular polymorphism, with granulocytes (neutrophils and eosinophils) lymphocytes and reticular cells.

In other words, of these three procedures, lymph node puncture was the easiest and the safest way to insure a diagnosis of Hodgkin's disease.

JPS

LYMPHOMAS AND LEUKEMIAS. THE VALUE OF EARLY DIAGNOSIS AND TREATMENT. L. F. Grater. From Memorial Hospital, New York. *J. A. M. A.* 136: 244-249, 1948.

This article is part of a series on early diagnosis and treatment of various forms of cancer. The author summarizes present principles of treatment of the lymphomatous disorders. A few highlights are:

1. Any inflammatory lymphadenopathy which persists for three weeks should be considered malignant until disproven by thorough investigation, including biopsy.
2. The treatment of choice for Hodgkin's disease or lymphosarcoma is intensive x-ray therapy to the involved areas. It is emphasized that the minimum doses just sufficient to cause regression of masses are not adequate therapy, but much larger doses—probably to the limit of skin tolerance—are indicated. (Specific schedules are given.)

3 The treatment of choice for chronic leukemias is also x-ray

4 The nitrogen mustards are of palliative value in certain disorders, notably generalized Hodgkin's disease. Radioactive isotopes are of palliative value, notably radioactive phosphorus or sodium in chronic leukemia. Urethane may be effective in some chronic leukemias, but inconstantly (25 to 33 per cent of cases). None of these agents is considered the treatment of choice.

5 Certain early cases of Hodgkin's disease, which seem to be localized, may be cured by complete local excision followed by intensive postoperative irradiation, or, perhaps, by intensive local irradiation alone.

S E

RECENT ADVANCES IN TREATMENT OF LYMPHOMAS, LEUKEMIAS AND ALLIED DISORDERS. L. F. Graver. From the Memorial Hospital, New York. *Bull. New York Acad. Med.* 24, 3-25, 1948.

The author summarizes his own experience at the Memorial Hospital and that of other recent observers in a well organized review. Particular emphasis is placed on (1) improvement in palliative results in Hodgkin's disease, lymphosarcoma and chronic lymphatic leukemia by early detection of local lesions and treatment with roentgen irradiation, (2) use of nitrogen mustards in patients with generalized Hodgkin's disease, (3) use of P<sub>32</sub> in polycythemia and in chronic myeloid and lymphatic leukemia with minimal enlargement of spleen, liver and nodes. The limited application of urethane in some cases of leukemia and of stilbamidine in multiple myeloma is briefly discussed.

In looking to the future, the cure of some cases of Hodgkin's disease and lymphosarcoma is considered a possibility. If these diseases have a unicentric origin, sufficiently early obliterative roentgen therapy or even radical surgery might prove curative, in the author's opinion. Considerable hope is also held for more specific attack on these disorders by means of further modifications in radioactive isotope therapy and by discovery of more effective chemical agents.

L E Y

DISCUSSION. TREATMENT OF THE LYMPHADENOPATHIES. R. B. Scott, M. J. Stewart, E. L. Cohen, D. W. Smitbers, and J. D. N. Nabarro. *Proc. Roy. Soc. Med.* 40, 617-622, 1947.

Several points of interest are included in this informal discussion of the treatment of the lymphomatous diseases. The generally accepted doctrine that, on the whole, treatment does not influence duration of life, although it may affect comfort and economic usefulness during life, is supported by comparative data. Comparison of patients with Hodgkin's disease treated with x-ray (1939) and without x-ray (1923) showed no statistical difference in survival times, although improvement of symptoms following x-ray is well known. A similar result is noted in the leukemias and reticulososes. Scott points out that surgical excision of a localized group of nodes involved in Hodgkin's disease may allow permanent cure, and mentions 3 such personal cases. Isolated instances of such result are well known, although the procedure is accepted with hesitation by most.

It is of interest that splenectomy is recommended (by Scott) in the occasional selected case of Hodgkin's disease or leukemia in whom symptomatic hemolytic anemia or symptomatic thrombocytopenia supervenes. The principle that splenectomy is not necessarily contraindicated once the diagnosis of lymphomatous splenomegaly has been made, but that the operation, by affording relief, especially from a hemolytic process superimposed on the underlying disorder, is becoming increasingly recognized as good medical practice in properly selected cases.

S E

THE CLINICAL AND PATHOLOGIC EFFECTS OF THE NITROGEN AND SULFUR MUSTARDS IN LABORATORY ANIMALS. I. Graf, D. A. Karnofsky, V. B. Jager, B. Krschkesky and H. W. Smith. From the Departments of Physiology and Pathology, New York University College of Medicine. *Am. J. Path.* 24, 1-47, 1948.

The effects of various nitrogen and sulfur mustards were investigated in albino mice, albino rats and New Zealand rabbits. Whenever possible, the effect of these compounds in other species was presented. In general, leukopenia was the most distinctive feature of the intoxication. Bone marrow reacted by showing initial degenerative changes followed by a rapid depletion of all hematopoietic cells. Weights of the spleens, thymuses and lymph nodes decreased markedly. These changes were reflected histologi-



cally by a loss of follicles in lymph nodes, an atrophy of malpighian corpuscles in the spleens and a shrinkage of the thymic cortex followed by involution. The effects of different routes of administration were discussed as well as the effects on other systems and tissues

O.P.J

THE EFFECTS OF  $\beta,\beta'$ -DICHLORODIETHYL-METHYLAMINE HYDROCHLORIDE ON THE BLOOD FORMING TISSUE  
G. R. Cameron, F. C. Courtice and R. P. Jones. From the Experimental Station, Porton, Wilts, England.  
J. Path. & Bact. 59: 425-435, 1947

A nitrogen mustard in the form of its hydrochloride was administered to rabbits and dogs for the purpose of studying its effect on the blood and hematopoietic organs. Doses given either intravenously or subcutaneously produced similar results. There was a generalized damage to the blood forming tissue but they did not all respond in the same manner nor to the same degree. Germinal centers of the lymph nodes and the spleen showed necrosis as early as three to six hours after administration of the drug. This was reflected in the blood by a lymphopenia and in the thoracic duct lymph by a decreased output. Damage to the bone marrow was reflected by a granulocytopenia following an initial increase. This granulocytopenia could be maintained by repeated doses of the drug. Since red corpuscles have a longer life than granulocytes in the circulating blood, anemia was produced only after repeated doses of the nitrogen mustard. Experimental anemias were produced in treated and untreated animals to determine the extent of bone marrow damage. The reticulocyte response was delayed in treated animals but it rose quite rapidly after the drug was discontinued. The action of this nitrogen mustard on the hematopoietic organs is very rapid and the recovery from it equally so.

O.P.J

NITROGEN MUSTARD AS A THERAPEUTIC AGENT FOR HODGKIN'S DISEASE, LYMPHOSARCOMA, AND LEUKEMIA  
M. M. Wintrobe, C. M. Huguley, Jr., M. T. McLennan, and L. P. de C. Lima. From the Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah. Ann. Int. Med. 27: 529-540, 1947

Seventy-seven patients were treated with di(B-chlorethyl) methylamine hydrochloride, employing in most cases a dose of 0.1 mg./kilo repeated four to six times. In Hodgkin's disease along with general improvement and decrease in size of the involved lymphoid tissue, there was a striking alleviation of fever. Pruritus was variably affected, dependent on the general response to treatment. Response was considered to be good in 61 per cent of 28 cases, and remission occurred in several patients considered to be x-ray resistant. In lymphosarcoma, response was good in 4 of 11 patients and in chronic leukemia one-third of the cases responded well. Results in acute leukemia were generally unsatisfactory although mention is made that small doses of mustard were very effective in bone pain. The authors observed a consistent reduction in both granulocytes and lymphocytes following therapy, but in only one case did they encounter the clinical picture of agranulocytosis. The red count and platelet levels were variably affected.

C.A.F

NITROGEN MUSTARD THERAPY IN CUTANEOUS BLASTOMATOUS DISEASE. E. D. Osborne, J. W. Jordan, F. C. Hoak, and F. J. Pschierer. From the University of Buffalo School of Medicine, Buffalo, New York.  
J. A. M. A. 135: 1123-1128 (1947)

This report concerns the effect of the nitrogen mustards in 4 patients with lymphomatous disorders of the skin, and 1 patient with diffuse lupus erythematosus. Two patients with mycosis fungoides showed prompt and dramatic improvement in both signs and symptoms, but rapid relapse with progression (and death in 1 case) occurred. One patient with lymphosarcoma showed dissolution of skin lesions, lymph nodes, and abdominal masses, but died within two weeks with progressive severe leukopenia. It is of interest that no gross or microscopic evidences for lymphosarcoma were present at autopsy, although biopsy before treatment showed definite lymphosarcoma. In a patient with combined Kaposi's sarcoma and Hodgkin's disease, the nitrogen mustard therapy had no effect. In a patient with chronic diffuse lupus erythematosus, who had had no response to x-ray therapy, the use of nitrogen mustard was followed within four days by virtual disappearance of the eruption. The patient continued to improve,

although a few areas of chronic discoid lupus erythematosus appeared on the face and neck within three months

Most dramatic in the patients' responses was relief of the severe, stubborn itching present in mycosis fungoides and in the patient with lupus. The results here presented duplicate, in general, other observations of the response of lymphomatous disorders to the nitrogen mustards. The authors emphasize that they do not advocate the general use of these agents in nonmalignant conditions such as disseminated lupus.

S E

EFFECTS OF URETHANE IN THE TREATMENT OF LEUKEMIA AND METASTATIC MALIGNANT TUMORS J S Hirschboeck, M C F Lindert, J Chase, and T L Calvy From Marquette University School of Medicine, Milwaukee County Hospital, Milwaukee, and Veterans Hospital, Wood, Wisconsin J A M A 136 90-95, 1948

The results of the treatment of 25 patients with leukemia, and 8 patients with malignant neoplasm, with urethane are summarized. The drug was most effective in producing remission in patients with chronic myelogenous leukemia. Its effect was inconstant in chronic lymphatic leukemia. It was of no value in the acute leukemias, or in the patients with carcinoma or sarcoma. These results are in line with previous investigations of the effects of urethane in these disorders. The drug is recommended as an adjunct form of therapy in selected cases of leukemia, and not as a general substitute for x-ray therapy in these diseases.

S E

LYMPHOBLASTOMA AN EVALUATION OF THE DIFFERENCES IN SENSITIVITY TO X-RAY IRRADIATION OF DIFFERENT TYPES, AND ITS APPLICATION TO A QUANTITATIVE THERAPEUTIC TEST W L Palazzo From the Radiation Therapy Department, Bellevue Hospital, New York Radiology 48 484-492, 1947

In this report the author attempts to classify various lymphomata according to their response to x-ray therapy, and proposes a therapeutic test with x-rays. His list places giant follicular lymphoblastoma at the top as most radiosensitive, and follows it with lymphatic leukemia, lymphosarcoma, polymorphous-cell sarcoma, and Hodgkin's disease.

A therapeutic test is recommended for deep-seated tumors in which biopsy is not feasible, such as mediastinal masses. In such tumors, the author believes, lymphatic leukemia is easily excluded by examination of blood and bone-marrow preparations. If this diagnosis has been ruled out, he then measures the size of the mass on an x-ray film, and then gives 600 roentgens to the lesion in two days, and re-x-rays and measures the mass three days later. If there has been a decrease in the size of the mass of 25 per cent or more, a diagnosis of giant follicular lymphoblastoma or of lymphosarcoma is in order, and a tumor-killing dose of x-ray is given. Recurrence after this suggests lymphosarcoma.

If the second x-ray examination shows no diminution in the size of the tumor, the author gives further irradiation to the tumor. If regression occurs when 1200 to 2000 roentgens have been given, the mass is considered a polymorphous-cell sarcoma. If regression occurs only after 2000 to 3000 roentgens, the mass is probably Hodgkin's disease. If no response has occurred after 3,000 roentgens have been given, the mass is radioresistant and probably belongs to a heterogeneous category including neurogenic sarcoma, carcinoma, various cysts, etc.

There is no discussion in the article of other forms of therapy of lymphomata, such as the nitrogen mustards.

S E

L ANEMIE DE LA MALADIE DE HODGKIN (The anemia of Hodgkin's disease) Georges Marchal (Paris) Rev Hemat 2 479-497, 1947

Anemia is generally considered as a sign of slight importance in the course of Hodgkin's disease. Marchal, on the contrary, considers it as an important factor and one of the essential signs of malignity. He has made a very comprehensive and well documented study of it.

Clinically, the author distinguishes three forms: anemias of an acute type, anemias connected with bony involvement, and anemia with splenomegaly. Clinical, anatomic and experimental studies show

that the mechanism of these anemias is essentially hemolytic (shown by the reticulocytosis and the indirect bilirubinemia) and also erythrophagic (shown by the macrophage hyperplasia found particularly in the spleen)

J.P.S

## IMMUNOHEMATOLOGY

PREPARATION FROM HUMAN RED CELLS OF A SUBSTANCE INHIBITING VIRUS HEMAGGLUTINATION P. M. de Burgh, Pen-Chung Yu, C. Houe, and M. Bovarnick From the Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass J. Exper. Med. 87: 1-9, 1948

Methods are described for extraction and purification of a substance inhibiting the agglutination of red cells by influenza (PR8) and mumps viruses. Human red cells of all types served as the chief source of inhibitor, activity being associated with the clinin fraction rather than with the stromatin fraction described by Calvin and coworkers. Material having similar properties was also found in human lung, but not in human liver, kidney or serum.

Active extracts were purified to the extent that 0.1 gamma of material inhibited one hemagglutinating dose of virus. The most highly purified fractions contained 2.6 per cent nitrogen, at least 50 per cent of polysaccharide, and no phosphorus. In the ultracentrifuge the purified preparation behaved as a polydisperse macromolecular substance. The active material was obtained from red cell stroma in an ether and chloroform-soluble form which, on further treatment, was converted into a chloroform insoluble form. It is considered possible that the former represents more closely the virus receptor as it exists in the intact cell.

The purified inhibitor was inactivated on incubation with virus at 37 C.

This report, and many others in the recent literature, serve to emphasize the importance of virus red cell relationships. It seems likely that further attempts to purify the various substances present in erythrocyte stroma may produce results that will aid in clarifying the nature of viral parasitism. The possible therapeutic implications of these studies are also apparent.

L.E.Y

## IRON AND PORPHYRIN METABOLISM

STUDIES ON THE TRANSPORTATION AND METABOLISM OF IRON IN THE BODY C.-B. Laurell From the Central Laboratory, University Hospital, Lund, Sweden Acta physiol. scand. Vol. 14, Suppl. 46, 1947

The work begins with a discussion of previous results regarding the transportation of iron. A short survey of the importance of the serum iron fraction is also given, together with a discussion of the iron binding capacity of serum. In this connection, the author mentions some of his own experiments aiming at an isolation of the specific iron-binding protein in the serum.

Holmberg's and Laurell's earlier work on the saturation limit for iron in the serum shows that there is a mean value of 312 gamma per cent for this limit, which agrees quite well with the mean maximal serum iron concentration of 291 gamma per cent found by Waldenström after the intravenous injection of iron into normal subjects. The iron-binding capacity of the serum is usually not fully utilized. The difference between the actual amount of serum iron and the saturation limit is called latent capacity. Above this saturation limit, the iron is loosely bound and reacts with phenanthroline directly. After a discussion of the different methods invented and used by the author, he finds that an indirect method is the easiest and best for the determination of the saturation limit. In the next chapter, the mode of binding of iron that has been added to the serum in vitro is discussed. With the aid of a number of ingenious adsorption experiments, the author shows that up to a certain concentration, iron is firmly bound and cannot be adsorbed. This holds true for both ferric and ferrous iron. The natural occurrence of ferric or ferrous iron linked with the serum protein cannot be determined. A large number of dialyzing experiments against serum seem to show that iron above the saturation limit is easily dialyzable and thus loosely bound. This would explain why the toxic effects of intravenously injected iron are seen only when the ceiling observed by Waldenström was reached. This ceiling thus corresponds to the saturation limit.

In some later experiments it was shown that the iron may be liberated from the natural serum iron complex without changing the capacity for iron-binding of this protein. The process is thus reversible.

The mean value for the saturation limit in a number of normal persons was found to be  $315 \pm 33\%$  per cent. Ingestion of iron does not increase the iron-binding capacity of the serum and the author concludes that the iron must leave the mucosa in ionized form and not as an iron-protein complex.

During the latter part of pregnancy, the values for the serum iron show a steady rise. There is a drop immediately before parturition. A study of maternal and fetal blood gives some interesting pieces of information. It is obvious that the iron-binding component of the maternal serum cannot pass freely through the placenta.

In posthemorrhagic anemia, the saturation limit is high but the serum iron values low. In untreated pernicious anemia, the mean saturation values were lowered ( $236\%$  per cent). The saturation limit was not affected by the rapid drop in serum iron under treatment. The limit was low in acute infections and paralleled the drop in the serum iron value. Cirrhosis of the liver and uremia showed a very low limit.

The work ends with a discussion of the theoretic implications of these results.

In a later paper published with Ingelman in *Acta chem. scand.*, the author has given a more detailed account of his preparative work. Swine serum was used for the experiments as it shows very little color from bilirubin. It is therefore easier to use the color intensity of the iron binding protein as an indicator for further fractionations. After addition of ferrous salts the color of the serum becomes reddish. Ammonium sulphate was added up to 60 per cent saturation. The precipitate was separated and about 90 per cent of the iron-binding component was still present in the solution. When the salt concentration was increased to 75 per cent, albumin and the iron containing protein came down as a precipitate, it was redissolved and dialyzed free from salt at pH 5.2 at  $0^\circ\text{C}$ . Ethyl alcohol was added to give a concentration of 20 volumes per cent. The solution was brilliantly red after the removal of the precipitate. After further precipitation of a new fraction, the solution of the iron containing protein was left at  $-15^\circ$ , when this protein was precipitated as a red sediment. Judging from its solubility in concentrated electrolyte solutions this protein cannot be regarded as a globulin. On electrophoresis, the isoelectric point was found to be about 4.4. At ultracentrifugation, the sedimentation constant was found to be 5.8. The diffusion constant was  $5.8 \times 10^{-7}$ . The parcel specific volume was not determined and the value given by Cohn and colleagues for their iron binding  $\beta$ -globulin was used for the calculation of the molecular weight. It was found to be 88,000. This agrees very well with the value given by Cohn for the metal-combining protein, but the isoelectric point was found by Cohn to be 5.6.

J. W.

**SERUM IRON LEVELS IN ADOLESCENT GIRLS. A STUDY OF THREE CASES.** *F. A. Johnston* From the Department of Home Economics, University of Chicago, Chicago, Ill. *Am. J. Dis. Child.* 74: 716-721, 1947.

The concentration of iron in the serum was determined eleven times over a period of five months in 3 adolescent girls whose well controlled diet contained 4 to 5 mg., then 9 mg. and 11 mg. iron per day for the three successive periods of the study. This investigation was carried out with the expectation that changes in serum iron on a controlled diet might provide an estimate of the iron requirement for growth and menstruation.

Hemoglobin levels of the subjects were near average values for girls, while the mean serum iron concentrations of 72, 62 and 46 micrograms per 100 cc. respectively were low, thus suggesting that serum iron might be more sensitive than hemoglobin as an index of iron stores. Since serum iron levels did not fall significantly, however, over a nine week period on low iron intake or rise over a period of twelve weeks on a fair iron intake, it was concluded that serum iron concentration does not respond to dietary intake quickly enough for use as a criterion of adequacy in short-term studies.

L. E. Y.

**A MICROMETHOD FOR THE QUANTITATIVE DETERMINATION OF THE URINARY COPROPORPHYRIN ISOMERS (I AND III).** *S. Schwartz, V. Haukinson, S. Cohen, and C. J. Watson*, From the Department of Medicine, University of Minnesota Hospital, Minneapolis, Minn., and the Metallurgical Laboratory, University of Chicago, Chicago, Ill. *J. Biol. Chem.* 168: 133-144, 1947.

A method is described for the extraction of coproporphyrin quantitatively from 100 cc. aliquots of urine and for its measurement fluorophotometrically. There are two alternate procedures for further purification and for isomer analyses, both dependent on the difference in fluorescence stability of the two porphyrin methyl esters in 30 to 35 per cent aqueous acetone in the cold.

C. A. F.

**PORPHYRIA AND PORPHYRINURIA** REPORT OF A CASE, REVIEW OF PORPHYRIN METABOLISM WITH A STUDY OF CONGENITAL PORPHYRIA I *Dunsky, S Freeman and S Gibson* From the Children's Memorial Hospital and the Department of Physiology, Northwestern University Medical School, Chicago, Ill Am J Dis Child 74 305-320, 1947

A case of congenital porphyria in a 17 month old girl is presented and its significance as related to an understanding of normal and pathologic pigment metabolism is emphasized. The clinical features of the case included red urine, erythrodontia, hydrot-aestivale and hirsutism. Analysis of the urine revealed the presence of uroporphyrin I and coproporphyrin I in addition to an unidentified porphyrin, the methyl ester of which melted at 209° to 212°C. The feces contained coproporphyrin I and another porphyrin with a somewhat higher melting point, but no uroporphyrin I was detected in the feces. A concise review of the literature on porphyrin metabolism is included in the report.

L.E.Y

## HEMOGLOBINURIA

**THE INFLUENCE OF AVAILABLE FLUID ON THE PRODUCTION OF EXPERIMENTAL HEMOGLOBINURIC NEPHROSIS IN RABBITS** J J *Lalich* From the Department of Pathology, University of Wisconsin Medical School, Madison, Wisconsin J Exper Med 87 157-162, 1948

Available fluid was measured by the thiocyanate method before and after a three day period of water deprivation in 20 rabbits, 15 of which were then given nine or ten intravenous injections of hemoglobin, totalling 1.8 Gm per Kg over a three day period. Wide variation in available fluid was found in different animals, especially in the females, some of which had appreciably lower volumes than any of the males. Wide variation was also noted in response to water deprivation. Inverse relationship is claimed between quantity of available fluid and severity of hemoglobinuric nephrosis, but the number of animals in each category is too small to permit conclusions in this respect. It should be noted, moreover, that during the period of hemoglobin injection all of the rabbits that urinated had an acid urine. The deleterious effect of dehydration might have been more clearly established if alkalinity of the urine could have been maintained.

Intravenously administered hemoglobin solutions were found to exert a diuretic effect in 3 of 7 rabbits tested, but when available fluid was depleted, elimination of hemoglobin appeared to be delayed or inhibited. It is postulated that under such circumstances, hemoglobin enters the renal tubules, pigment casts are formed, degeneration of tubular epithelium appears and uremia follows.

Additional findings of interest were necrosis of the liver and pulmonary edema in some of the rabbits that died of uremia. The combined weight of the kidneys of rabbits dying of hemoglobinuric nephrosis was significantly in excess of that of the control rabbits and of the test animals with transient nephrosis.

L.E.Y

**RENAL ATROCYTOSIS AND INTRACELLULAR DIGESTION OF INTRAPERITONEALLY INJECTED HEMOGLOBIN IN RATS** L J *Rather* From the Department of Pathology, Stanford University School of Medicine, San Francisco, Calif J Exper Med 87 163-174, 1948

Rats were injected intraperitoneally with solutions of human hemoglobin in total doses averaging 5 Gm per Kg. Serum hemoglobin concentration, rate of excretion of hemoglobin in the urine and urine output were measured, and the rats were then killed at varying intervals for the purpose of studying the tissues.

In rats killed two hours after the first injection of hemoglobin, discrete bodies with the staining characteristics (Dunn's stain) of hemoglobin were found in small numbers within the epithelium of the proximal convoluted tubules. In rats killed at seventeen hours, the renal epithelium was packed with these particles, and it was found that formation of casts did not occur to any extent until the amount of atrocytosed material reached a maximum. Although it was inferred that break-up of the hemoglobin began prior to release of inorganic iron, the appearance of hemosiderin was the first visible evidence of intracellular splitting of hemoglobin. Deposits of hemosiderin were most marked at about sixty five hours after which time they slowly disappeared. It is of interest that very little hemosiderin and no hemoglobin were demonstrable in the parenchymal or Kupffer cells of the liver, nor in phagocytic cells of spleen or femoral marrow.

There was no evidence that intracellular accumulation of hemoglobin damaged renal epithelium, and it was not until large amounts of hemoglobin were present within lumina of the nephrons that injury could be detected. Oliguria was attributed to interference with flow through lumina due to increased viscosity of protein-rich fluid.

These observations are in general agreement with those reported by other investigators and they support the hypothesis that hemoglobin filtered by glomeruli undergoes athrocytosis by tubular cells and is then returned to the blood in a simpler form.

L E Y

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# CONVENTION NOTES

## INTERNATIONAL SOCIETY OF HEMATOLOGY

BUFFALO, N Y, AUGUST 23-26, 1948

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THE International Society of Hematology is to hold its first formal meeting in Buffalo, August 23-26, 1948. Conceived in Dallas, Texas, in 1946 during the first part of an International Rh and Hematology Conference, the Society began to assume form when the decision to go ahead with its organization was made during the second part of the Conference in Mexico City in November 1946. The guiding spirits of the new organization were Drs. Joseph M. Hill and Sol Haberman of Dallas, Texas. It was fitting, therefore, that these two were later elected president and secretary respectively. Dr. Eduardo Uribe Guerola, of Mexico, was elected Vice-President and Dr. W. Stuart Stanbury, of Canada, was elected as treasurer.

It was decided that hematology had become of sufficiently impelling interest to so many individuals throughout the world that an organization devoted to this interest was fully justified. It was believed that the convening of different groups of individuals—clinicians, pathologists, chemists, immunologists and the like—would offer distinct benefits to all concerned. It was felt that the gathering of individuals from different parts of the world would help to foster important relationships and to break down the barriers of the printed word. For these reasons a truly International Society was envisaged.

A *membership committee* was appointed consisting of the following individuals

William Dameshek, Chairman Boston, Mass.	Moises Chediák Havana	Alfredo Pavlovsky Buenos Aires, Argentina
R. Philip Custer Philadelphia, Pa.	Walter Cruz Rio de Janeiro	Robert R. Race London
O. P. Jones Buffalo, N. Y.	Henrik Dam Copenhagen	Karl Rohr Zurich
Roy Kracke Birmingham, Ala.	C. R. Das Gupta Calcutta	Luis Sandoval S. Santiago, Chile
Philip Levine Raritan, N. J.	D. diGuglielmo Naples	Rod Sirivejkul Bangkok, Siam
M. Bessis Paris	I. González-Guzmán Mexico, D. F.	Theodore Waugh Montreal
Berger Broman Stockholm	Ludwik Hirszfeld Wrocław, Poland	

It is planned to have at least two categories of members, senior and associate, but the final regulations for membership have not as yet been completed. It is believed that each country or group of countries should be free to carry out its own membership admissions in a more or less autonomous manner, subject to general

rules to be decided upon at the first meeting of the *constitution committee* The latter committee is composed of the following

Israel Davidsohn, Chairman  
Chicago

I Gutierrez Villegas  
Mexico, D F

A H T Robb Smith  
Oxford, England

Joseph Ross  
Boston

L M Tocantins  
Philadelphia

## PROGRAM NOTES AND NEWS

The meeting promises to be a landmark in the field of hematology not only because it will mark the first truly International Congress in that field, but because of the enthusiastic interest being shown by people from all over the world All those interested in hematology are invited to this meeting Headquarters will be at the Hotel Statler, Buffalo, New York All inquiries regarding program, exhibits, etc are to be forwarded to Dr Sol Haberman, Secretary For further information, see box on page 725

The program committee for the Buffalo meeting is as follows

Ernest Witebsky, Chairman  
Buffalo

Louis K Diamond  
Boston

Carl Moore  
St Louis

Walter Seegers  
Detroit

L H Snyder  
Norman, Oklahoma

Eduardo Uribe Guerola  
Mexico, D F

Lionel Whitby  
Cambridge, England

### TENTATIVE PROGRAM

Day 1	One-half day	Methods including phase microscopy, electron microscopy, and histo-chemical methods
	One-half day	Hemorrhagic diseases
Day 2		Red cell problems Anemia
Day 3		Hemolytic Anemia and Immunohematology
Day 4		White cell problems Leukemia, Lymphoma

In addition to the speakers listed on the next page, it is planned to hold a certain number of round table discussions

Dr Oliver P Jones of Buffalo, the chairman of the committee on exhibits has announced that a number of exhibits both professional and commercial are in process of preparation A preliminary list of exhibits and exhibitors is given on pages 724-726 Other Committees and Chairmen appointed for the Meeting are *Accommodations*, L Edgar Hummel, M D , Buffalo, N Y , *Publicity*, David K Miller, M D , Buffalo, N Y , *Banquet*, Douglas P Arnold, M D , Buffalo, N Y , *Local Information*, E George Phillies, M D , *Registration*, Stuart L Vaughan, M D , *Women's Activities*, Mrs Mildred Lockwood, *Excursion and Entertainment*, Charles Becker, M D , *Equipment* (Co-chairmen), Siegfried Tannhauser, M D , and James F Mohn, M D



## SOME OF THE SPEAKERS AND THEIR SUBJECTS

- Oliver P Jones, University of Buffalo, Buffalo, New York  
*Newer Interpretations of Nuclear Pattern of Immature Blood Cells*
- L B Jaques, University of Saskatchewan, Saskatoon, Saskatchewan, Canada  
*Studies on the Silicone Technic for Delaying Blood Coagulation*
- L K Diamond, Harvard Medical School, Children's Hospital, Boston, Mass  
*Congenital Absence of Fibrinogen*
- Clement A Finch, Peter Bent Brigham Hospital, Boston, Mass  
*Iron Metabolism in Hemochromatosis*
- John G Gibson, 2nd, Harvard Medical School, Boston, Mass  
*Determination of the Zinc Content of Normal Human Erythrocytes, Leucocytes, Plasma and Whole Blood*
- R G MacFarlane, Radcliffe Infirmary, Oxford, England  
*Problems of Coagulation and Hemorrhagic Diseases*
- Elbert deCoursey, Brooke General Hospital, San Antonio, Texas  
*Effects of Lethal Instantaneous Ionizing Radiation upon the Reticulo-endothelial System*
- M Hynes, Cambridge University, Cambridge, England  
*The Iron Reserves of a Normal Man*
- Floyd Daft, National Institute of Health, Bethesda, Maryland  
*Interchangeability of Folic Acid, Other Vitamins and Amino Acids in the Correction of Experimental Blood Dyscrasias*
- A H T Robb-Smith, Radcliffe Infirmary, Oxford, England  
*The Effects of Transfusion Hemosiderosis*
- D Shemin, Columbia University, New York City  
*The Biologic Synthesis and Metabolism of Porphyrins*
- Walter H Seegers and Arnold G Ware, Wayne University, Detroit, Michigan  
*Activation of Purified Prothrombin*
- Robin Coombs, University of Cambridge, Cambridge, England  
*Observations on Some of the Immunological Processes Concerned in the Diagnosis and Manifestations of Hemolytic Disease of the Newborn*
- R R Race, Lister Institute, London, England  
*Blood Groups and Their Inheritance with Particular Reference to the Rh and MN Systems*
- J J van Loghem, Binnengasthuis, Amsterdam, Holland  
*ABO Incompatibility and Rh Antagonism*
- G Failla, Columbia University Medical Center, New York City  
*Correlation of Radiation Dose and Effect on Hemopoietic System*
- Maurice Bessis, Paris, France  
*Exchange Transfusion in Diseases Other than Hemolytic Disease of the Newborn*
- W Jacobson, School of Medicine, University of Texas, Corpus Christi, Texas  
*The Nature of the Anti-pernicious Anemia Factor and the Enzymatic Activation of Folic Acid*
- J V Dacie, London, England  
*Hemolytic Mechanism and Laboratory Diagnosis of Nocturnal Hemoglobinuria*

Eugene C Loomis, Parke Davis & Co , Detroit, Michigan

*Antifibrinolysin, Biochemical Concentration of Fibrinolysin and anti-fibrinolysin*

J P Soulier, France

*Resegnements fournis par l'action de la thrombine sur les plasmas ayant un temps de Quick allonge*

M Burstein, France

*Plaquettes et hemophilia*

Allen F Reid and Frances Jones, Baylor Hospital, Dallas, Texas

*New Method for Fractionation of Plasma Proteins for Therapeutic Use*

Luis Gutierrez Villegas, Mexico, D F , Mexico

Subject to be announced

Alfredo Pavlovsky, Buenos Aires, Argentina

*Contribution to the Pathogenesis of Hemophilia Study of the Vascular Factor*

Lawrence E Young—University of Rochester, Strong Memorial Hospital,  
Rochester, New York

*Hemolytic Reactions Produced in Dogs by Transfusion of Incompatible Dog Blood*

Ludwik Hirszfeld, Wroclaw, Poland

*The Pleiades Concept of the ABO Blood Antigens*

Mourant, A E , Lister Institute, London, England

*The Rh Group of the Basques and the Origin of the d-Gene in Europe*

Douglas P Arnold, Buffalo, New York

*Treatment of Hemolytic Disease of the Newborn*

John Tinsley and Carl Moore, Washington University, St Louis, Missouri

Subject to be announced

John Olwin, Presbyterian Hospital, Chicago, Ill

*The Control of Dicumarol Therapy with Remarks on the Ac Globulin Factor*

Phil Levine, Ortho Research Foundation, Raritan, New Jersey

Subject to be announced

Ernest Witebsky, Buffalo General Hospital, Buffalo, New York

Subject to be announced

# LIST OF EXHIBITORS AT THE INTERNATIONAL

Bethell, Frank, Simpson Memorial Institute,  
Ann Arbor, Michigan Co-exhibitors Drs  
Gould Andrews, Murial Meyers and Rosalie  
Neligh

Calhoun, Lois, Department of Anatomy, School  
of Veterinary Medicine, Michigan State Col-  
lege, East Lansing, Michigan

Charipper, Harry, Department of Biology,  
Washington Square College, New York Uni-  
versity, New York City, N Y Co exhibitor  
Dr Albert S Gordon

Davidsohn, Israel, Chicago, Ill , and Kurt Stern  
and Miss Chiyo Kashiwagi

Dougherty, Thomas F , University of Utah, Salt  
Lake City, Utah

Ervin, Donald, University of Rochester, Roch-  
ester, New York Co-exhibitors Dr L E  
Young, Dr D Ervin, Dr Edward Von Has-  
selt and Dr J E Thomas •

Fallon, Madeleine, Los Angeles, California

Guest, George, Children s Hospital, Cincinnati,  
Ohio

Haberman, Sol, and Hill, M J , Baylor Hospital,  
Dallas, Texas

Hall, Byron E , Mayo Clinic, Rochester, Minn  
Co-exhibitors Charles Stroebel, C H Watkins  
and W A Bennett

Hargraves, Malcolm M , Mayo Clinic, Roches-  
ter, Minn Co-exhibitors Frank Heck and  
E D Bayrd

Hawn, C V Z , Basset Hospital, Cooperstown,  
N Y , and Porter, K R , Rockefeller Institute  
for Medical Research, New York, N Y

Isaacs, Raphael, Chicago, Illinois

Jacobson, Leon O , Billings Hospital, Chicago,  
Illinois Co-exhibitor Dr M H Block

Jacques, Louis, University of Saskatchewan,  
Saskatoon, Saskatchewan

Jones, Oliver P , and Richards, O W Univer-  
sity of Buffalo and American Optical Com-  
pany

I Bone Marrow Examination by Several Tech-  
niques

II Blood and Marrow Changes Observed Fol-  
lowing the Administration of Pteroylgly-  
camic Acid

Normal Bone Marrow of Domestic Animals

The Endocrine System and Hemopoiesis

The Serologic Diagnosis of Infectious Mononu-  
cleosis

Title to be announced

Hemolytic Transfusion Reactions I Destruction  
of A1 and A2 cells by transfusion of blood from  
a dangerous universal donor Dr Donald Ervin  
and Dr Lawrence E Young II Hemolytic re-  
actions produced in dogs by transfusion of in-  
compatible dog blood (IIA) Immunologic and  
hematologic aspects, (IIB) Renal aspects  
Charles L Yuile, Christine Waterhouse, Henry  
Tesluk, James A Bush and John W Hayden

Bone Changes in the Anemias in Infancy and  
Childhood, Delayed Ossification and Growth  
Retardation, Changes in the Size and Shape of  
the Heart

Osmometric Behavior of Red Blood Cells (a)  
Normal and abnormal types of human erythro-  
cytes, (b) normal cells of various species  
Studies on the Reactivity of Antibodies with  
Specific Emphasis on the Cryptagglutinoids  
Radioactive Phosphorus

Marrow Findings in Lupus Erythematosus and  
Multiple Myeloma

Effect of Various Agents on the Leukocytes in  
treatment of Leukemia

Effect of Induced Anemia on Radiation Damage

The Use of Silicone in Controlling Coagulation  
of Blood

Phase Microscopy in Hematological Research

# HEMATOLOGY CONGRESS AND PROPOSED TITLES

Kastlin, George J , Pittsburgh, Pa

Knisely, Melvin H , Department of Anatomy,  
University of Chicago, Chicago, Illinois  
Limarzi, Louis R , Chicago, Illinois, and Drs  
Carrol L Birch, Raymond S Kibler, Jerome T  
Paul, Robert M Jones and Paul L Bedrogi  
Meyer, Leo O , and C G Grand

Mohn, James, Department of Bacteriology, Uni-  
versity of Buffalo, Buffalo, New York  
Muirhead, E E , Baylor Hospital, Dallas, Texas  
Noonan, Thomas R , Atomic Energy Project,  
University of Rochester, Rochester, New  
York Co-exhibitors Dr K Altman, Dr K  
Solomon, Mr G A Boyd, Mr G W Casarett  
Osgood, Edwin E , Department of Medicine,  
University of Oregon Medical School, Portland,  
Oregon

Ralph, Paul H , Department of Anatomy, Uni-  
versity of Washington, Seattle, Washington  
Rebuck, John, Henry Ford Hospital, Detroit,  
Michigan

The Effect of Nitrogen Mustard on X-ray Resist-  
ant Cases of Leukemia and Lymphoblastoma  
Method for Studying Circulating Blood in the  
Human Bulbar Conjunctiva  
Multiple Myeloma

Tissue Culture Studies with Myelokentric and  
Lymphokentric Acids  
Blood Group Specific Substances

Acute Renal Insufficiency and its Management  
The Contribution of Tracer Techniques to He-  
matology

The biological half life of radioactive phosphorus  
in various types of cells, tissues and body fluids  
in patients with leukemia and polycythemia  
treated with radioactive phosphorus  
Title to be announced

Electron Microscope Studies of Blood Cells

(Continued on next page)

## INFORMATION

- First formal meeting, INTERNATIONAL SOCIETY OF HEMATOLOGY
- AUGUST 23-26, 1948
- BUFFALO, N Y , Headquarters, STATLER HOTEL
- All those interested in hematology may attend

REGISTRATION FEE Ten dollars\*

For information regarding program, exhibits, etc , contact Dr Sol Haberman,  
Secretary, 3301 Junius St , Dallas, Texas

For information regarding hotel reservations, contact Dr Edgar Hummell,  
University of Buffalo, Buffalo, N Y , Chairman of the Accommodations  
Committee See reservation information and form, page 727

\* Interns and residents and guests from those countries which at present are having a dollar shortage (i e certain European and Asiatic countries) will be exempt from the registration fee. This fee likewise does not apply to sponsors and those presenting scientific exhibits at the Congress.

## LIST OF EXHIBITORS AT THE INTERNATIONAL HEMATOLOGY CONGRESS AND PROPOSED TITLES (*Continued*)

Rubinstein, Michael A , New York, New York	Aspiration of Bone Marrow from the Iliac Crest
Schwartz, Steven O , Chicago, Illinois Co-exhibitors Dr W J Kawula and Dr S R Kaplan	Thrombocytopenic Purpura
Singer, Karl, Michael Reese Hospital, Chicago, Illinois	Studies on the survival time of red cells in health and disease
Smith, Carl, 103 E 84th St , New York, N Y	Outpatient Transfusion Clinic for Ambulatory Patients with Mediterranean and Other Chronic Anemias
Stanbury, W S , Calgary, Alberta	'Miracle Fluid' (film)
Sundberg, Dorothy, Institute of Anatomy, University of Minnesota, Minneapolis, Minn	Granulomatous Lesions in Bone Marrow Obtained by the Aspiration Method
Tompkins, Edna H , Laboratory of Applied Physiology, Yale University, New Haven, Conn	Avoidance of Errors in Use of Hayem's Fluid
Ware, Arnold G , Department of Physiology, Wayne University College of Medicine, Detroit, Michigan Co-exhibitor Dr John Fahey	The 2 stage Analysis for Prothrombin and Ac globulin
Wiener, Alexander S , Brooklyn, New York	Rh-Hr Blood Types and the Heredity—also the Rh Antibodies and their mode of action
Young, Lawrence E , University of Rochester, School of Medicine and Dentistry, Rochester, New York Co-exhibitor Dr D Ervin	Hemolytic Transfusion Reactions (Destruction of A1 and A2 cells by transfusion of blood from a dangerous universal donor)

## COMMERCIAL EXHIBITS

In addition to the preceding list of scientific exhibits, a variety of commercial exhibits is planned. The following list of exhibitors is incomplete, as requests for exhibit space were still being received as this goes to press.

Vend All Limited

Lederle Laboratories

Cutter Laboratories

Armour Laboratories

Hospital Liquids Incorporated

Technicon Company

Bausch & Lomb Company

Grune & Stratton

The Society is obtaining financial assistance from several sources to allay the expenses of the Congress. These financial contributors are tentatively listed in two classes. Patrons, who have contributed \$1,000 or more, Sponsors, who have contributed between \$500 and \$1,000. The following list, to which additions may be expected, is complete as of press time.

### PATRONS

Lederle Laboratories Division, American

Cyanamid Company, New York, N Y

Hoblitzelle Foundation, Dallas, Texas

University of Buffalo, Buffalo, N Y

Parke, Davis & Company, Detroit, Mich

### SPONSORS

Armour Laboratories, Armour & Company, Chicago, Ill

Hospital Liquids, Incorporated, Chicago, Ill

American Optical Company, Scientific Instruments Division, New York, N Y

## INFORMATION FOR HOTEL ACCOMMODATIONS

For your convenience in making hotel reservations for the coming meeting of the International Society of Hematology, August 23-26, 1948, in Buffalo, New York, hotels and their rates are listed below. The meetings are to be held at the Hotel Statler and a total of 350 rooms are being held in reserve. However, those attending the meetings may apply for rooms at any of the hotels they desire, all of which, in the list below, are relatively near and convenient to the convention hotel. Those planning to attend from countries other than the United States and Canada may, if they choose, fill in the form below, detach and return as directed. The local committee will then make reservations as near to their indicated wishes as possible. *Such request should be sent in time to be received in Buffalo not later than August 1, 1948, and must give the date and hour of arrival as well as definite date and approximate hour of departure, names and addresses of all persons who will occupy reservations must be included.* The Hotel Statler, upon request, is anxious to send a questionnaire card to those interested in obtaining reservations at the convention hotel.

### RATES (Minimum)

	<i>Single</i>	<i>Double</i>	<i>Twin</i>
Hotel Buffalo, Washington and Swan Sts	\$2 50 up	\$4 50 up	\$5 00 up
Hotel Lafayette, Washington and Clinton Sts	3 50 up	6 50 up	7 00 up
Hotel Lenox, North near Delaware Ave	4 00 up	6 00 up	6 00 up
Hotel Markeen, Main at Utica St	2 25 up	3 50 up	
Hotel Richford, 210 Delaware Ave	2 00 up	3 50 up	5 00 up
The Sheraton, 715 Delaware Ave	3 50 up	6 00 up	6 50 up
Hotel Statler, Delaware Ave at Niagara Sq	3 50 up	5 75 up	7 00 up
Hotel Stuyvesant, 245 Elmwood Ave	3 50 up	5 50 up	
Hotel Touraine, 274 Delaware Ave	3 50 up	5 50 up	7 00 up

For tourist information and resort accommodations, write to Buffalo Convention & Tourist Bureau, Inc., Buffalo 2, New York, U S A

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(TO BE USED ONLY BY THOSE ATTENDING FROM COUNTRIES OTHER THAN  
THE UNITED STATES AND CANADA)

ALL RESERVATIONS MUST BE RECEIVED NOT LATER THAN AUGUST 1, 1948

L E HUMMEL, M D

Chairman, Committee on Accommodations

537 Delaware Avenue

Buffalo 2, New York, U S A

Please reserve the following accommodations for the meeting of the International Society of Hematology, to be held in Buffalo, N Y, August 23-26, 1948

Single Room	Double Bedded Room	Twin Bedded Room
Rate From \$	to \$	First Choice Hotel
		Second Choice Hotel
		Third Choice Hotel

Arriving at Hotel (date)                      Hour                      A M                      P M                      Leaving

THE NAME OF EACH HOTEL GUEST MUST BE LISTED. Therefore, please include the names of both persons for each double or twin bedded room requested.

Names and addresses of all persons for whom you are requesting reservations and who will occupy the rooms asked for

If the hotels of your choice are unable  
to accept your reservation the Com-

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# BOOK REVIEWS

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*Pathologisch-anatomische Untersuchungen über Leberzirrhose bei Säuglingen und Kleinkindern (infantile Leberzirrhose) mit endemischer Häufung* By HERMANN GÖGL Vienna, Wilhelm Maudrich, 1947

This monograph, by Dr Hermann Gögl of Innsbruck, is an interesting study of 24 cases of infantile cirrhosis in which the general picture is that of a chronic hepatitis with diffuse cirrhosis in varying degree. Other changes serving to characterize these cirrhoses from a histologic standpoint are the presence of eosinophilic and sometimes basophilic granules within the liver cells, and at times of inclusion-like bodies, suggesting a virus infection. Gögl points out that this disease is common in the Tyrol and Vorarlberg regions of Austria, but more particularly around the town of Kitzbühl where it seems to have endemic character. He obviously leans toward a virus etiology of his own cases but for lack of evidence is obliged to leave the question open as to exact etiologic agents and the relation to epidemic hepatitis.

In addition to the careful clinical, anatomic, and histologic descriptions, the latter supplemented with reasonably satisfactory photomicrographs, there is an extensive bibliography on the subject of cirrhosis in infancy and early childhood.

C J WATSON

*Der Blutspender* By H. WILLENEGGER AND R. BOITEL Benno Schwabe & Co., Basel (Switzerland), 1947  
Pp 197, 10 francs

This is a unique work, as books about blood transfusions and blood banks go. The literature to date has emphasized blood groups and transfusion reactions, the management of blood banks and the latest discoveries arising from physico-chemical manipulations of whole blood and its constituents. This book, however, treats of the blood donor, the fountainhead and mainstay of all this activity and the forgotten man of most publications. The uniqueness of the point of view alone would justify the writing of such a book. This small volume, however, has even more to recommend it. Although the main interest centers about the blood donor and the social significance of his service in contemporary society, the scope of the work is wider and comprises many of the problems accompanying the therapeutic use of blood and plasma.

An illustrated historical account of the development of blood transfusions opens the book and is carried up to modern times and the use of fresh and reconstituted plasma. There follows an excellent discussion of the individual blood donor, his physical and psychologic examination, the question of payment, and the problem of transmission of disease from donor to recipient, with special emphasis on syphilis. The blood groups are briefly but adequately presented in a clearly diagrammed manner, although the Rh complex is summarily treated in one short paragraph and presents the state of knowledge of early 1942. The major and most interesting portion of the book deals with the rise of blood donor organizations in modern society. It stresses the social aspects of the problem of an adequate supply of blood donors in the face of mounting use of blood transfusions. The development of blood donor societies in each of the major countries is individually outlined and the types of organizations are discussed and criticized.

This book is written in a simple style, almost as if it were intended more for the general lay public than for the medical profession. However, it does give an excellent general review of the whole problem of an adequate source of supply of human blood, and the methods, both organizational and psychologic, by which such a source might be set up. It is certainly advantageous reading for all those physicians actively associated with blood banks and blood transfusion services.

J. NEBER

# BLOOD

## *The Journal of Hematology*

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VOL III, NO 7

JULY, 1948

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### RELATION OF ADRENAL CORTICAL HORMONE TO LYMPHOID TISSUE AND LYMPHOCYTES

B<sub>3</sub> WILLIAM N VALENTINE, M D , CHARLES G CRADDOCK, JR , M D , AND  
JOHN S LAWRENCE, M D

#### GENERAL INTRODUCTION

THE CONCEPT of hormonal control of the numbers of the circulating blood lymphocytes is not new. The remarkably narrow range of fluctuation of blood cells under normal circumstances has long stimulated scientific investigators to speculate over the manner of their regulation. The possibility of a humoral mechanism has intrigued many. The literature pertinent to humoral theories of regulation of the blood lymphocytes has been reviewed by Drinker and Yoffey<sup>22</sup> in their monograph published in 1941. At the time of the review, no humoral theory had gained wide acceptance.

Within the last few years, interest in the hormonal control of the blood lymphocytes has been stimulated by a large number of reports suggesting the pituitary-adrenal cortical control of lymphoid tissue structure and function, including the regulation of the numbers of circulating lymphocytes. It has been postulated that the secretion of the adrenal cortex (more specifically the sugar hormone) is the normal determinant of the numbers of blood lymphocytes. Fitted into the broader pattern of the alarm reaction and adaptation syndrome of Selye, offering as it does new vistas of the physiology of lymphoid tissue, and illuminating the poorly understood functions of the lymphocyte, this concept demands the careful and critical scrutiny of all the facts on which it is based.

It is the object of this report (1) to present experimental data on the effect of adrenal cortical hormone on the numbers of thoracic duct lymphocytes and on the rate of flow of thoracic duct lymph in the normal and adrenalectomized cat, (2) to briefly present data on the peripheral blood counts of cats before and after adrenalectomy, (3) to recapitulate in considerable detail as much as possible of the actual published data relating to hypophyseo-adrenal cortical control of lymphoid tissue structure and function and of the numbers of blood lymphocytes. To this latter end the second part of this communication is devoted to critical review of the subject,

From the Department of Medicine of the University of Rochester School of Medicine and Dentistry, Rochester, New York.

This paper is based on work performed under Contract No. W-7401-eng-49 with the Atomic Energy Commission for the University of Rochester, Rochester, New York.



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#### GENERAL INTRODUCTION

THE CONCEPT of hormonal control of the numbers of the circulating blood lymphocytes is not new. The remarkably narrow range of fluctuation of blood cells under normal circumstances has long stimulated scientific investigators to speculate over the manner of their regulation. The possibility of a humoral mechanism has intrigued many. The literature pertinent to humoral theories of regulation of the blood lymphocytes has been reviewed by Drinker and Yoffey<sup>22</sup> in their monograph published in 1941. At the time of the review, no humoral theory had gained wide acceptance.

Within the last few years, interest in the hormonal control of the blood lymphocytes has been stimulated by a large number of reports suggesting the pituitary-adrenal cortical control of lymphoid tissue structure and function, including the regulation of the numbers of circulating lymphocytes. It has been postulated that the secretion of the adrenal cortex (more specifically the sugar hormone) is the normal determinant of the numbers of blood lymphocytes. Fitted into the broader pattern of the alarm reaction and adaptation syndrome of Selye, offering as it does new vistas of the physiology of lymphoid tissue, and illuminating the poorly understood functions of the lymphocyte, this concept demands the careful and critical scrutiny of all the facts on which it is based.

It is the object of this report (1) to present experimental data on the effect of adrenal cortical hormone on the numbers of thoracic duct lymphocytes and on the rate of flow of thoracic duct lymph in the normal and adrenalectomized cat, (2) to briefly present data on the peripheral blood counts of cats before and after adrenalectomy, (3) to recapitulate in considerable detail as much as possible of the actual published data relating to hypophyseo-adrenal cortical control of lymphoid tissue structure and function and of the numbers of blood lymphocytes. To this latter end the second part of this communication is devoted to critical review of the subject,

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it being hoped that the assemblage of the available experimental data will better permit a dispassionate analysis of the present status of the hormonal control of lymphocytes and lymphoid tissue

## PART I

### A EXPERIMENTAL STUDIES ON THE EFFECT OF ADRENAL CORTICAL EXTRACT ON THE NUMBERS OF LYMPHOCYTES AND RATE OF FLOW OF THORACIC DUCT LYMPH

#### *Introduction*

Only two previous reports are available on the effects of adrenal cortical extract or adrenocorticotrophic hormone on the output of thoracic duct lymphocytes in the experimental animal. The first of these, a short preliminary communication by Reinhardt and Li,<sup>56</sup> reported marked reduction in the number of thoracic duct lymphocytes in rats following the subcutaneous or intraperitoneal injection of 1 to 6 mg. of pure adrenocorticotrophic hormone. This was apparent within fifteen to thirty minutes after injection and lasted four to ten hours. No data are presented on the number of animals used or the findings in individual animals. The common lymph sacs of rats anesthetized with sodium pentobarbitol were intermittently cannulated in this group of experiments and leukocyte counts obtained at thirty to sixty minute intervals for several hours before and after the administration of hormone. The authors conclude that "it may be generalized that the lymphocyte level of the circulating blood is under direct adrenal cortical control."

In 1946, observations by Yoffey, Reiss, and Baxter<sup>73</sup> on the influence of adrenocorticotrophic hormone on the rate of lymph flow and numbers of lymphocytes in thoracic duct lymph appeared in the British literature. These investigators found a decrease in the lymphocyte count and volume of flow of thoracic duct lymph in cats after administration of 300 units of adrenocorticotrophic hormone. In the data presented from a single, typical experiment flow reduction from 2 to 3 cu. cm. an hour to 1 cu. cm. an hour was observed. Lymphocyte counts were reported as having fallen to somewhere below 10,000 per cu. cm. two to three hours after hormone administration, the pre-injection count being in the neighborhood of 50,000 to 60,000 lymphocytes per cu. cm.

The measurement of the influence of adrenal cortical extract on numbers of thoracic duct lymphocytes entering the blood stream is a particularly pertinent part of the investigation of the hypothesis of adrenal cortical control of lymphoid tissue structure and function and of the numbers of circulating lymphocytes. Although there has been some speculation, there is virtually no evidence of an abnormal rate of disappearance of lymphocytes from the peripheral blood under the influence of increased adrenal cortical activity. The literature in regard to adrenal cortical regulation of blood lymphocyte levels will be carefully examined in the second part of this report. Suffice it to say that the latter postulate rests heavily upon the histologic and functional alterations produced by the sugar hormone of the adrenal cortex in lymphoid structures themselves. The available evidence lays emphasis on involutionary changes wrought by adrenal cortical activity on the factories manufacturing lymphocytes, on the temporary cessation of lymphocyte production, and

on the dissolution of lymphocytes within the lymphoid tissues. It should then be possible to obtain confirmation or refutation of alterations in the rate of delivery of lymphocytes to the blood by examination and study of the volume of lymph and numbers of lymphocytes in thoracic duct lymph at the point of its entrance into the venous circulation. Although not all of the lymphocytes enter the blood by this route, it is such a preponderant and major source of blood lymphocytes that it appears reasonable to feel that any widespread interference with lymphocyte production would be reflected by changes in thoracic duct lymph. The present data in the literature are fragmentary and scanty. This investigation was therefore undertaken with the hope of adding direct observations on the delivery of lymphocytes to the systemic circulation by thoracic duct lymph before and after the injection of adrenal cortical hormone.

### *Experimental Approach and Methods*

Although direct observation of lymphocytes in thoracic duct lymph is an appealing approach and offers many advantages, the problem of control is a difficult one. First, it is widely recognized that the numbers of lymphocytes in any given drop of lymph may vary tremendously in the same animal. Second, it is also a matter of record that thoracic duct lymphocyte production may show very wide variations from one animal to another. Age, general health, individual peculiarities and numerous other considerations of undetermined nature are all probably important in this regard. Third, lymph flow is influenced by many factors such as the time relationships with feeding, the nature of ingested food, the variations in muscular tone induced by varying depths of anesthesia, etc. Fourth, the mechanical factors incident to cannulation of the thoracic duct and the mere fact that lymph is lost from the body may well result over a period of hours in qualitative or quantitative alterations of the lymph. These alterations may conceivably make their appearance only after a considerable interval of time. Fifth, physiologic increase in the activity of the intact adrenals of the experimental animal may well occur as a result of the alarming stimulus of the operative procedure of thoracic duct cannulation itself. An alarm reaction may mask the effect of injected hormone and may vary in intensity from animal to animal.

With these considerations in mind it is apparent that each cannulated animal receiving adrenal cortical extract must have all comparisons made in terms of *its own* baseline determinations obtained prior to injection of hormone. It is not possible to quantitatively compare absolute values for thoracic duct lymphocytes of one animal to another. However, if in each animal one expresses these values for each given time interval after injection as a ratio or percentage of pre-injection values, it is possible to compare these *ratios or percentages* from animal to animal. Since in each animal the evaluation of the effect of hormone depends upon relatively few observations, it is highly important in this type of experiment to compare values obtained from large quantities of lymph so that the wide variations in numbers of cells present in any one drop of lymph may be minimized. Since the effect of hormone is determined for each animal individually the necessity for complete uniformity of all factors in all animals is to a large extent mitigated. It is important, however, that enough experiments be done to determine the presence or absence of any significant trend and that the experiments be of sufficient duration to cover the period when effects would be expected.

It is also apparent that if the effects of the procedure itself are to be ruled out, a control group of animals not injected with hormone but handled in an identical manner as the experimental group must be obtained. Valid comparisons can only be made between values obtained in experimental and control animals at the same interval of time after thoracic duct cannulation. Changes noted without benefit of a control group could otherwise easily be due to the procedure itself. If the possibility of adrenal activity incident to an alarm reaction is to be ruled out as an uncontrolled variable, the experimental animal must include an adrenalectomized group. An attempt was made in so far as possible to control the effects by the following experimental procedure.

The experimental animal employed was the cat. Animals were divided into three groups. Group I consisted of control normal animals which were to receive no injections of adrenal cortical hormone. Group

II consisted of normal animals which were to receive injections of adrenal cortical hormone. Group III consisted of bilaterally adrenalectomized cats which were likewise to receive injections of adrenal cortical extract. In all groups studied the same procedure was followed. Under anesthesia induced by the intraperitoneal injection of sodium pentobarbital, the thoracic duct was exposed at its entrance into the venous circulation in the neck. Whenever the presence of a single main duct permitted it, cannulation of the thoracic duct itself was performed, a small glass cannula being inserted into the duct and held in place by a ligature. Any small subsidiary ducts were tied off. In a few instances where multiple small ducts entered a segment of vein the latter was isolated from all connection with the circulation and cannulated. Not all animals of course can be successfully cannulated. Also, in some instances small collateral ducts must be ligated and there is some doubt that lymph flowing from the cannula is completely equivalent to the entire volume of lymph normally entering by the complete ductal system. However, it certainly constitutes the major portion of the original total flow, and since results in each animal are referred only to baseline determinations in the same animal this is not an important point. It is important that a satisfactory cannulation and reasonably adequate lymph flow be obtained in each experiment. It was customary to place a small amount of 1 per cent sodium heparin in the cannula to prevent any clotting of lymph while cannulation was in progress. On completing the procedure the animal was turned in the prone position and placed on a wooden support. A small oval aperture in this support under the cannulated duct permitted the latter to hang down at a roughly 45 degree angle and to deliver its lymph by dripping freely into a collecting tube. The cannula was allowed to drip for a few minutes before collection was started in order to clear itself of heparin and to relieve any abnormal backlog of lymph which might have accumulated in the ductal system while cannulation was being performed.

The collecting container consisted of a glass centrifuge tube calibrated so that lymph volumes could be directly measured. This was placed under the cannula and contained three drops of a 1 per cent solution of pure sodium heparin (Abbott) to assure that no clotting of the lymph occurred. Lymph was collected in hourly volumes, in the majority of experiments for an eight hour period. The hourly volumes were recorded and the specimens then thoroughly mixed for five minutes. Leukocyte counts were usually made in triplicate on each specimen. In a very few instances only duplicate counts were made and in similarly few instances more than three counts were made. The counts could be checked within reasonably close limits. From the average leukocyte values per cubic millimeter and the volume it was of course possible to compute the number of white cells delivered per hour from the cannula. Smears of the lymph or its centrifuged sediment were made in every case and stained with Wright's stain. From the differential cell counts determined from such smears, the total leukocytes per hour were readily converted into absolute lymphocytes per hour. In addition to the above, one or more total and differential leukocyte counts were made on the peripheral blood during the experiment. This was done to insure that no animal used was suffering from infectious feline agranulocytosis. The hematologic studies were all made by one or another of the authors and only Bureau of Standards certified equipment was used. It was customary to maintain fluid and electrolyte balance by subcutaneous injections of isotonic saline during the experiment. Body temperature was maintained by the warmth of an electric light placed a few inches above the animal.

In normal and adrenalectomized animals receiving injections of adrenal cortical extract, two hourly collections of lymph were obtained as a baseline prior to injection. The body weight of the animals employed in this group of studies varied from 2.1 kilograms to 4.75 kilograms. Only 2 animals weighed more than 4 kilograms. No attempt was made to administer adrenal cortical extract per kilogram of body weight. However, large doses of adrenal cortical extract (Upjohn) were employed since it was desired to assure that changes, should they be found to occur, be as nearly maximal as possible. The smallest dosage given any cat, therefore, on a kilogram for kilogram basis would have been equivalent to the administration of about 145 cc. of the same preparation to a 70 kilogram man. In the normal group of 5 animals receiving hormone, 2 animals received 10 cc., 2 animals received 20 cc. and 1 animal received 25 cc. In the adrenalectomized group of 9 animals, 1 animal received 10 cc., 4 animals received 15 cc., 2 animals received 20 cc., and 2 animals received 25 cc. All injections were made subcutaneously into the tissues of one or the other rear extremities.

In the adrenalectomized group of animals, the adrenal glands were removed in two stages through flank incisions. Aseptic precautions were maintained and no studies were made on any animal developing wound infection. In order that the effects of the operative procedure would be negligible, no thoracic duct cannulations were performed until from two to twelve days after the removal of the second adrenal

gland The average interval between adrenalectomy and cannulation was five and two-thirds days In all animals used, the wound appeared healing normally or well healed During this period, animals were maintained in good condition by the following regimen Immediately after the second adrenalectomy, each animal received a single subcutaneous injection of adrenal cortical extract (Upjohn), an intramuscular injection of 5 mg (1 cc) of desoxycorticosterone in peanut oil (Percorten, Ciba) and subcutaneous isotonic salt solution No further adrenal cortical extract was given thereafter, but each day 2.5 mg (0.5 cc) of desoxycorticosterone in peanut oil was administered intramuscularly No medication was given the day of thoracic duct cannulation Desoxycorticosterone in dosage of this magnitude has been demonstrated repeatedly to have little or no effect on lymphoid tissue structure or function A solution containing 0.7 per cent sodium chloride and 0.3 per cent sodium bicarbonate was substituted for drinking water in the cages If an animal appeared dehydrated, sterile isotonic saline was administered under the skin In this manner, animals could be maintained in satisfactory shape for operation At the conclusion of the cannulation experiment, each animal was autopsied and in no case was macroscopic adrenal tissue demonstrable

The animals in these experiments were fed as usual the day before the procedure It was also customary to place a supplementary dish of milk in the cage late the afternoon before an animal was to be used No food was given the morning of the cannulation

The methods of handling the data obtained are discussed with the presentation of the data

### *Presentation of Data*

Data are presented on 9 control animals, 5 normal animals administered adrenal cortical extract two hours after thoracic duct cannulation, and 9 bilaterally adrenalectomized animals also administered adrenal cortical extract two hours after cannulation

The data presented in tables 1, 2, and 3 indicate the lymph volume in cubic centimeters, the absolute lymphocyte counts per cubic millimeter of lymph of each specimen, and the total number of lymphocytes in each hourly collection of lymph for each cat These figures are presented for the purpose of completeness and in order that the full picture in each animal will be available for each hour after cannulation On the basis of this data, analyses for significant correlation between the treated and untreated groups of animals have been made in the following manner In all groups, the findings of the first two hours have been used as a standard of reference This standard of reference has been computed in two ways In one set of determinations the *average* values for the first two hours have been set as equal to 100 per cent In another set of determinations the values for the second hour only have been set as equal to 100 per cent The findings in each subsequent hour are then expressed as a percentage of those employed for the standard of reference This has been done in each animal, separate ratios having been determined for each of the two standards of reference mentioned before Ratios are presented for lymph volume in cubic centimeters, absolute lymphocytes per cubic millimeter, and total lymphocytes for each hourly collection period For example, if the average lymph volume in a given cat for the first two hours was 10 cc per hour and the third hour's flow was 8 cc, then the flow during the third hour was eighty per cent of the standard of reference The flow during each succeeding hour could be similarly expressed If, in the same animal, the second hour's flow was 12 cc, the flow during the third hour in terms of this standard of reference would be 66.6 per cent Ratios of absolute lymphocytes per cubic millimeter and total lymphocytes can be determined and expressed in an identical manner Separate sets of figures are presented for each stand-



ard of reference merely to determine whether any correlations could be found using either baseline. It was felt that perhaps the values obtained during the second hour might be considered more useful because the animal has had an opportunity by that time to adjust to the procedure. On the other hand the average of the first two hours

TABLE 1.—*Lymph Volumes per Hour, Absolute Lymphocyte Counts per Cu Mm of Each Hourly Specimen and Total Lymphocytes per Hour of Thoracic Duct Lymph in a Control Group of Normal Untreated Cats*

Hour after Can- nu- lation	Variable	Cat Number									
		445	462	474	483	499	506	523	532	544	592
1	Lymph vol in cc	7.2	8.8	6.4	8.8	8.3	10.1	12.5	4.4	3.2	14.0
	Lymphs per cu mm	28,850	19,800	14,949	10,909	22,383	6402	8316	18,414	18,834	13,167
	Total lymphs/hr, $\times 10^6$	207.7	174.2	95.7	96.0	185.8	64.7	104.0	81.0	60.3	184.3
2	Lymph vol in cc	5.2	5.1	6.0	8.6	5.9	6.7	8.3	4.4	3.5	7.2
	Lymphs per cu mm	22,180	15,427	6883	10,033	9050	4702	7343	9883	12,375	13,150
	Total lymphs/hr, $\times 10^6$	115.3	78.7	41.3	86.3	53.4	31.5	60.9	43.5	43.3	94.7
3	Lymph vol in cc	5.8	4.9	6.8	6.6	6.0	7.4	9.5	3.7	4.2	6.7
	Lymphs per cu mm	16,269	10,081	8811	8464	13,017	4290	6014	10,400	17,584	12,900
	Total lymphs/hr, $\times 10^6$	94.4	49.4	59.9	55.9	78.1	31.7	57.1	38.5	73.9	86.4
4	Lymph vol in cc	5.8	4.9	4.6	7.2	6.0	8.2	7.5	4.7		5.0
	Lymphs per cu mm	18,980	18,583	17,150	9440	4900	4655	5917	11,333		16,089
	Total lymphs/hr, $\times 10^6$	110.1	91.1	78.9	68.0	29.4	38.2	44.4	53.3		80.4
5	Lymph vol in cc	2.0	3.9	4.5	6.0	7.3	8.4	7.1	3.8	2.4	4.4
	Lymphs per cu mm	25,823	12,544	9042	7856	7000	4785	9517	9538	9533	13,933
	Total lymphs/hr, $\times 10^6$	51.6	48.9	40.7	47.1	51.1	40.2	67.6	36.2	22.9	61.3
6	Lymph vol in cc		4.2	3.8	5.5	5.7	7.7	8.6	3.7	2.9	4.7
	Lymphs per cu mm		10,518	8496	9247	6833	4767	6027	8184	6548	9625
	Total lymphs/hr, $\times 10^6$		44.2	32.3	50.9	38.9	36.7	51.8	30.3	19.0	45.2
7	Lymph vol in cc		3.3	4.5	5.5	6.7	7.5	6.8	3.8	4.2	4.1
	Lymphs per cu mm		17,100	7178	7778	8467	5032	9338	12,756	11,217	12,625
	Total lymphs/hr, $\times 10^6$		56.4	32.3	42.8	56.7	37.7	63.5	48.5	47.1	51.8
8	Lymph vol in cc		3.0	4.2	5.4	6.7	6.1	6.3	2.7	3.2	
	Lymphs per cu mm		13,365	8016	10,064	11,632	7333	5760	9250	10,183	
	Total lymphs/hr, $\times 10^6$		40.1	33.7	54.3	77.9	44.7	36.3	25.0	32.6	

has the advantage of being a larger baseline. Both computations are therefore presented. It likewise seemed wise to seek correlations between as many different factors as possible, and therefore lymph volume, lymphocytes per cubic millimeter, and total lymphocytes were all investigated in this manner. One or both of the first two hours were used as a baseline because in no group was any animal administered adrenal cortical extract during the first two hours. In the treated groups of both normal and adrenalectomized animals each cat received adrenal cortical extract at the end of the second hour.

Having computed the ratios for each animal in these ways, it was possible to compare the animals within each group at any given hour after cannulation. Thus, for example, suppose hypothetically that in an adrenalectomized group of 3 cats,

TABLE 2.—Lymph Volumes per Hour, Absolute Lymphocyte Counts per Cu. Mm. of Each Hourly Specimen and Total Lymphatics per Hour of Three Different Lymph in a Group of Normal Cats Administered Adrenal Cortical Extract after a Two Hour Baseline

Hour after Cannulation	Variable	Cat Number				
		101	105	401	422	463
1	Lymph vol. in cc	4.3	15.0	8.5	10.6	5.5
	Lymphs per cu. mm	15,305	14,933	8,498	8180	5309
	Total lymphs/hr	$65.8 \times 10^6$	$224.0 \times 10^6$	$70.5 \times 10^6$	$86.7 \times 10^6$	$29.2 \times 10^6$
2	Lymph vol. in cc	5.9	11.6	6.4	10.4	5.6
	Lymphs per cu. mm	15,650	8135	4243	6345	4818
	Total lymphs/hr	$92.3 \times 10^6$	$94.4 \times 10^6$	$27.2 \times 10^6$	$66.0 \times 10^6$	$27.0 \times 10^6$
Adrenal Cortical Extract Administered						
3	Lymph vol. in cc	6.1	9.7	7.9	9.7	4.3
	Lymphs per cu. mm	16,583	8850	4843	5275	4354
	Total lymphs/hr	$101.2 \times 10^6$	$85.8 \times 10^6$	$38.3 \times 10^6$	$51.2 \times 10^6$	$18.7 \times 10^6$
4	Lymph vol. in cc	5.8	8.0	7.8	7.7	3.5
	Lymphs per cu. mm	16,764	9372	5428	4313	4105
	Total lymphs/hr	$97.2 \times 10^6$	$75.0 \times 10^6$	$42.3 \times 10^6$	$33.2 \times 10^6$	$14.4 \times 10^6$
5	Lymph vol. in cc	5.7	7.3	7.4	6.8	4.2
	Lymphs per cu. mm	17,917	15,461	4884	5433	3224
	Total lymphs/hr	$102.1 \times 10^6$	$112.9 \times 10^6$	$36.1 \times 10^6$	$36.9 \times 10^6$	$13.5 \times 10^6$
6	Lymph vol. in cc	4.3	6.5	5.5	5.8	3.8
	Lymphs per cu. mm	21,383	14,421	4925	5102	4105
	Total lymphs/hr	$91.9 \times 10^6$	$93.7 \times 10^6$	$27.1 \times 10^6$	$29.6 \times 10^6$	$15.6 \times 10^6$
7	Lymph vol. in cc	4.5	6.7	4.8	5.1	3.3
	Lymphs per cu. mm	16,813	8224	8901	6450	2945
	Total lymphs/hr	$75.7 \times 10^6$	$55.1 \times 10^6$	$42.7 \times 10^6$	$32.9 \times 10^6$	$9.7 \times 10^6$
8	Lymph vol. in cc	4.5	5.6	3.9	4.7	3.5
	Lymphs per cu. mm	13,530	7712	5183	4888	3322
	Total lymphs/hr	$60.9 \times 10^6$	$43.2 \times 10^6$	$20.2 \times 10^6$	$23.0 \times 10^6$	$11.6 \times 10^6$

ratios of lymph volumes during the third hour were 70, 80, and 90 per cent respectively. The mean value for such a group during the third hour would be 80 per cent of the standard of reference. Suppose that in a control group for the third hour the comparable mean is 90 per cent. The difference in the means for the two groups can be tested for significance. This can be done for each hour and for each factor to be compared. Significant differences or trends if they exist can be related to the admin-

istration of adrenal cortical extract at the end of the second hour in the treated groups, since the animals were otherwise handled in the same manner as untreated

TABLE 3—*Lymph Volumes per Hour, Absolute Lymphocyte Counts per Cu Mm of Each Hourly Specimen And Total Lymphocytes per Hour of Thoracic Duct Lymph in a Group of Bilaterally Adrenalectomized Cats Administered Adrenal Cortical Extract after a 2 Hour Baseline*

Hour after Can-nu-lation	Variable	Cat Number									
		423	436*	440	466	494	519	524	537	542	566
1	Lymph vol in cc	7 6	4 7	3 5	4 8	3 0	3 5	4 0	6 2	4 4	10 2
	Lymphs per cu mm	11,888	15,550	9374	11,715	31,083	28 207	32 250	33,533	28 075	14,602
	Total lymphs/hr, $\times 10^6$	90 3	73 1	32 8	56 2	93 2	98 7	129 0	207 9	123 5	148 9
2	Lymph vol in cc	5 7		2 8	4 4	3 0	3 0	4 5	6 0	4 5	7 9
	Lymphs per cu mm	10,000		4500	16 533	27,951	17 589	36 500	17,717	38 776	16 950
	Total lymphs/hr, $\times 10^6$	57 0		12 6	72 7	83 9	52 8	164 2	106 3	174 5	133 9

#### Adrenal Cortical Extract Administered

3	Lymph vol in cc	5 2	4 0	2 1	4 8	3 6	2 1	4 4	3 8		6 9
	Lymphs per cu mm	14,298	23 483	14,635	12,917	32,653	8967	39 983	20,250		11 583
	Total lymphs/hr, $\times 10^6$	74 3	93 9	30 7	62 0	117 6	18 8	175 9	77 0		79 9
4	Lymph vol in cc	4 9	3 4	2 5	4 6	3 5	3 5	3 5	6 0	4 0	5 6
	Lymphs per cu mm	13,899	22 117	7491	28,517	51 450	9787	37 250	21 533	30 070	8052
	Total lymphs/hr, $\times 10^6$	68 1	75 2	18 7	131 2	180 1	34 3	130 4	129 2	120 3	45 1
5	Lymph vol in cc	4 7	3 0	2 7	3 4	3 7	3 8	3 6	4 2	3 1	5 4
	Lymphs per cu mm	13,818	30,525	10 544	22 866	38 889	10,567	38 133	24 354	24,832	8051
	Total lymphs/hr, $\times 10^6$	64 9	91 6	28 5	77 7	143 9	40 2	137 3	102 3	77 0	43 5
6	Lymph vol in cc	5 7	2 8	2 8	1 1	3 9	4 2	3 9	3 6	2 9	5 1
	Lymphs per cu mm	9306	19,828	5033	27 083	38 083	10,626	23 056	15 680	32 093	8588
	Total lymphs/hr, $\times 10^6$	53 0	55 5	14 1	29 8	148 5	44 6	89 9	56 4	93 1	43 8
7	Lymph vol in cc	5 1	2 8	2 5	3 6	5 0	4 0	4 3	2 0	3 2	4 9
	Lymphs per cu mm	6928	19,632	5247	6402	33 891	7178	32 224	16,709	19 899	7631
	Total lymphs/hr, $\times 10^6$	35 3	55 0	13 1	23 0	169 5	28 7	138 6	33 4	63 7	37 4
8	Lymph vol in cc	5 8	3 7	2 8	3 2		3 5	4 6	1 1	2 5	5 0
	Lymphs per cu mm	11,067	17,968	4059	7234		10,133	34 898	18 576	19 333	5968
	Total lymphs/hr, $\times 10^6$	64 2	66 5	11 4	23 1		35 5	160 5	20 4	48 3	29 8

\* The data on cat number 436 are not included in Table 5 since data on the second hour specimen was not available

controls This has been done in tables 4 and 5 and the various factors tested for significance by the computation of "Student's" t-ratios and the corresponding 'P' or probability values<sup>51</sup>

The significance of the differences in the means for each factor at each interval of

time can be readily seen by looking at the 'P' means in each table. 'P' values of 0.01 to 0.05 are generally considered statistically significant. Values in the neighborhood of .10 may have questionable significance. When values much above this level are encountered there is considered to be no detectable significant difference between the compared factors.

TABLE 4.—*Results for the Significance of the Differences in Mean Lymph Volume per Hour Ratios, Mean Absolute Lymphocyte per Cu. Mm. Ratios, and in Mean Total Lymphocyte per Hour Ratios between a Control and a Normal + Cortex Group of Cats*

Hour after Treatment	Group	Basis	Lymph Volume Per Hour					Absolute Lymphocytes Per cu. mm					Total Lymphocytes Per Hour				
			mean	$\sigma$	$M_1 - M_2$	t Ratio	P <sub>mean</sub>	mean	$\sigma$	$M_1 - M_2$	t Ratio	P <sub>mean</sub>	mean	$\sigma$	$M_1 - M_2$	t Ratio	P <sub>mean</sub>
1	1*	First 2 hrs	10.88	6.18	5.1	50	.63	80.1	15.8	3.5	.42	.68	71.3	27.7	7.5	.49	.63
			5.93	7.19				83.9	13.8				78.8	28.9			
1	2	2nd hr only	10.102	2.11	6.1	71	.49	101.3	28.0	0.8	.06	.95	104.6	36.8	7.0	.37	.72
			5.96	1.18				100.5	13.1				97.6	28.5			
2	1	First 2 hrs	9.81	0.17	2.0	18	.86	91.1	35.0	7.8	.46	.65	70.1	24.7	0.2	.01	.99
			5.83	0.24				83.3	17.4				70.3	34.2			
2	1	2nd hr only	9.95	4.17	10.3	.93	.37	113.3	55.4	12.6	.48	.64	104.2	40.0	15.4	.67	.52
			5.85	1.24				100.7	24.0				88.8	43.4			
3	1	First 2 hrs	10.70	1.22	11.2	.89	.39	81.7	23.0	11.5	.83	.42	53.6	17.5	20.5	1.60	.13
			5.81	3.23				93.2	30.1				74.1	33.1			
3	1	2nd hr only	10.81	0.26	2.1	.15	.88	99.6	21.2	14.8	.86	.41	79.5	27.9	14.3	.83	.42
			5.83	1.22				114.4	47.0				93.8	38.1			
4	1	First 2 hrs	9.72	7.15	9.6	.77	.46	67.2	17.2	31.4	2.49	.03	47.5	15.3	17.5	1.48	.16
			5.66	1.14				98.6	30.7				65.0	29.8			
4	1	2nd hr only	9.84	1.18	16.4	1.77	.10	83.5	20.4	35.6	2.25	.04	69.9	22.2	10.3	.77	.46
			5.67	7.12				119.1	39.8				80.2	26.8			
5	1	First 2 hrs	9.75	9.26	13.7	1.05	.32	84.3	19.7	9.5	.68	.51	60.8	19.9	1.7	.13	.90
			5.62	2.15				93.8	32.9				59.1	30.1			
5	1	2nd hr only	9.86	0.23	22.6	1.97	.07	104.0	16.9	12.2	.63	.54	89.4	26.2	12.8	.66	.52
			5.63	4.11				116.2	55.5				76.6	48.0			
6	1	First 2 hrs	8.69	7.17	11.6	1.13	.28	82.0	22.4	8.0	.74	.48	55.7	19.0	12.3	1.12	.29
			5.58	1.18				74.0	10.3				43.4	19.9			
6	1	2nd hr only	8.78	1.19	19.5	2.01	.07	105.3	26.7	15.4	1.10	.29	84.5	38.0	31.7	1.74	.11
			5.58	6.12				89.9	20.5				52.8	16.6			

\*1 Control Group  $\sigma$  = Standard Deviation for each Mean

†2 Normal + Cortex Group  $M_1 - M_2$  = Difference in the two means i.e. Mean of Group 1 minus Mean of Group 2

NOTE: The first hour after treatment represents the *third* hour after cannulation in each group. The first two hours were employed as a baseline standard of reference. The control group was administered no adrenal cortical extract. The figures for the means are in every instance presented as a percentage of the mean of the standard of reference.

### Analysis and Discussion of Data

It can be clearly seen from tables 4 and 5 that under the conditions of these experiments no significant effect attributable to the injection of large doses of adrenal cortical hormone was demonstrable. Whether lymph flow, the number of lymphocytes per cubic millimeter of lymph, or the total number of lymphocytes per hour

are compared, there is no significant difference between the untreated animals, the normal animals administered adrenal cortical extract, or the adrenalectomized animals administered adrenal cortical hormone. Moreover, this is true whether the

TABLE 5 — *t* Ratios for the Significance of the Differences in Mean Lymph Volume per Hour Ratios, in Mean Absolute Lymphocyte per Cu Mm Ratios, and in Mean Total Lymphocyte per Hour Ratios between a Control and an Adrenalectomized + Cortex Group of Cats

Hour after Treatment	Group	Basis	N	Lymph Volume Per Hour					Absolute Lymphocytes Per cu mm					Total Lymphocytes Per Hour					
				mean	$\sigma$	$M_1 - M_2$	t Ratio	$P_{mean}$	mean	$\sigma$	$M_1 - M_2$	t Ratio	$P_{mean}$	mean	$\sigma$	$M_1 - M_2$	t Ratio	$P_{mean}$	
1	1*	First 2 hrs	10	88 6 18 3	8	84 5 21 8	4 1	43	67	80 4 15 8	26 0	1 53	15	71 3 27 7	89 4 41 4	18 1	1 11	28	
	3†	2nd hr only	10	102 2 14 3	8	89 2 19 5	13 0	1 63	12	101 3 28 0	125 8 86 0	24 5	85	41	104 6 36 8	109 3 64 7	4 7	19	85
2	1	First 2 hrs	9	81 0 17 6	9	90 0 17 3	9 0	1 09	29	91 1 35 0	109 7 52 4	18 6	89	39	70 1 24 7	101 2 61 5	31 1	1 41	18
	3	2nd hr only	9	95 4 17 1	9	94 5 16 1	0 9	11	91	113 3 55 4	118 5 51 1	5 2	21	84	104 2 40 0	114 6 59 1	10 4	44	67
3	1	First 2 hrs	10	70 1 22 8	9	83 8 22 1	13 7	1 33	20	81 7 23 0	105 5 42 0	23 8	1 56	14	53 6 17 5	87 9 42 4	34 3	2 35	03
	3	2nd hr only	10	81 0 26 6	9	88 2 22 6	7 2	63	54	99 6 21 2	118 2 57 6	18 6	95	36	79 5 27 9	105 7 60 8	26 2	1 23	24
4	1	First 2 hrs	9	72 7 15 9	9	81 2 34 5	8 5	67	51	67 2 17 2	89 3 45 8	22 1	1 36	19	47 5 15 3	66 4 40 3	18 9	1 32	21
	3	2nd hr only	9	84 1 18 3	9	85 6 36 3	1 5	11	91	83 5 20 4	94 5 37 2	11 0	78	45	69 9 22 2	77 9 45 3	8 0	48	64
5	1	First 2 hrs	9	75 9 26 4	9	87 2 39 2	11 3	72	48	84 3 19 7	66 3 25 6	18 0	1 67	11	60 8 19 9	61 7 53 1	0 9	05	96
	3	2nd hr only	9	86 0 23 8	9	91 4 39 0	5 4	35	73	104 0 16 9	74 0 32 4	30 0	2 46	03	89 4 26 2	70 5 55 8	18 9	92	31
6	1	First 2 hrs	8	69 7 17 7	8	73 9 30 5	4 2	34	74	82 0 22 4	65 6 24 3	16 4	1 40	18	55 7 19 0	49 5 33 0	6 2	46	65
	3	2nd hr only	8	78 1 19 1	8	78 8 32 6	0 7	05	96	105 3 26 7	73 5 30 0	31 8	2 24	04	84 5 38 0	58 6 38 0	25 9	1 36	20

\*1 Control Group

$\sigma$  = Standard Deviation for each mean

†3 Adrenalectomized + Cortex Group  $M_1 - M_2$  = Difference in the two means i.e. Mean of Group 1 minus Mean of group 2

NOTE The first hour after treatment represents the third hour after cannulation in each group. The first two hours were employed as a baseline standard of reference. The control group was administered no adrenal cortical extract. The figures for the means are in every instance presented as a percentage of the mean of the standard of reference.

first two hours after cannulation or the second hour only is used as a baseline standard of reference. If the 'P' or probability values for all factors and for both standards of reference are examined, only 8 out of the entire group are found to be below 10. In 3 of these 8, values for the treated groups were "significantly" higher than the values for the control group although the time interval after injection was

such that the opposite might have been expected. In 5 of the 8, values were higher in the control than in the treated group. In only one instance were "P" values of 10 or below obtained with *both* standards of reference. These are found in the fourth hour after injection (sixth hour after cannulation) when comparison is made of the mean absolute lymphocytes per cubic millimeter of the control group and the normal group receiving adrenal cortical extract. In this instance, the values for the treated group were above those for the control group and thus the reverse of what would be expected according to the hormonal hypothesis. In all other instances where apparently significant "P" values were obtained for one standard of reference they were not for the other. In short, the data in these experiments give no clue to any significant effect of adrenal cortical extract on the numbers of lymphocytes or rate of flow of thoracic duct lymph. No trend of any sort is detectable after hormone injection.

Although several factors were analysed, the total lymphocytes delivered by the thoracic duct per hour should, on theoretical grounds at least, have been the most important. Regardless of the numbers of lymphocytes per unit of lymph or the rate of lymph flow, as far as maintaining the numbers of blood lymphocytes is concerned, the pertinent factor is the total number delivered to the blood. Examination of the data shows no suggestion of a trend in this important factor.

Data are available for six hours after the injections of hormone (eight hours after cannulation) in the treated groups. This interval of observation was considered sufficiently long since in all species studied on which there are previous reports maximum depression of blood lymphocytes after the administration of adrenal cortical extract was found to occur within three to six hours after injection. The small amount of data available on thoracic duct lymphocyte levels after the administration of adrenal cortical hormone also suggested depression well within this time interval.

It should be noted that rate of flow of thoracic duct lymph in cannulated animals tends to diminish as time goes on. In measuring the effect of any factor on rate of flow a control group is therefore highly important. This diminution in flow is not unexpected since all the factors incident to the procedure including the loss of lymph from the body would predispose to reduction in the rate of lymph flow.

In addition to the experiments analysed in the tables, six additional experiments of the same type have been performed. In five of these, normal animals were injected with adrenal cortical extract after obtaining baseline determinations as a standard of reference, in one experiment, an adrenalectomized animal was similarly treated. These have not been included in the detailed analysis of the data because adrenal cortical extract was not administered until more than two hours after cannulation. Thus, hour by hour comparison with the major body of the experiments could not validly be made. However, examination of the data in these experiments likewise shows no demonstrable trend following the injection of hormone. They are essentially comparable to the data reported in detail in the tables.

It was of interest to note that significant changes were not apparent even in the adrenalectomized group of animals. These had presumably been deprived for a period of a few days of any effect of the sugar hormone of the adrenal cortex on

their lymphoid structures. It was thought that any effect of adrenal cortical extract on thoracic duct lymphocytes would be readily manifest in such a group.

### Conclusions

Under the conditions of these experiments on the cat and with the dosage and preparations of adrenal cortical hormone employed, it has been impossible to demonstrate significant hormonal effects on the numbers of lymphocytes or rate of flow of thoracic duct lymph.

TABLE 6 — *Average Value for White Blood Cells in Cats prior to and after Adrenalectomy*

Cat Number	W B C per cu mm		Stabs and Segmented Neutrophils per cu mm		Lymphocytes per cu mm	
	Prior to Adrenalectomy	After Adrenalectomy	Prior to Adrenalectomy	After Adrenalectomy	Prior to Adrenalectomy	After Adrenalectomy
1	15475	23686	9733	19013	4713	2978
2	20050	45391	10102	29863	4633	7277
3	17725	14600	7090	10181	8690	3008
4	16800	17631	7129	11838	7829	4321
5	17833	21404	9931	15304	7465	4060
6	20350	19031	15289	15251	4377	3279
7	14667	17543	10651	13649	2906	3091
8	12450	15116	7043	10025	4286	3955
9	18600	19483	8928	14300	8556	4304
10	16100	17515	11270	10995	3059	4730
11	11590	19241	7635	12734	3384	5838
12	25450	25282	17599	16931	5474	7332
13	23163	28790	15547	19999	7003	7734
14	18250	16025	11827	10318	6046	4748
15	9127	10900	4988	5917	3584	4413
Average	17175	20776	10318	14421	5467	4738

### B STUDIES ON THE BLOOD COUNTS OF CATS BEFORE AND AFTER ADRENALECTOMY

#### Presentation of Data

The following data indicate the total and differential leukocyte counts in fifteen cats before and after bilateral adrenalectomy. The studies reported were made by one of us (J. S. L.)<sup>12</sup> a few years prior to the present investigation. In every instance repeated determinations of the numbers and differential formula of the white blood cells in the peripheral blood were made prior to adrenalectomy. Blood was obtained from the ear vein, and determinations were made only with Bureau of Standards equipment. In two instances, one set of observations only was made. In all other cases three or more white blood cell studies were made during the control period and in eight of the fifteen animals five or more control observations were obtained. In practically all instances the blood studies were made at daily intervals during the postoperative period. All animals lived at least seven days after the removal of both adrenal glands and the average survival period was ten days. No animals received hormonal maintenance therapy after adrenalectomy. All animals developed the

typical picture of adrenal insufficiency prior to death and on postmortem examination no adrenal tissue was found in any case. Operations were aseptically performed and at autopsy trivial skin or wound infections were found in only 2 animals (numbers one and eight) and osteomyelitis of the tail in one animal (number five). The values given in the table are the *averages* of the observations made before and after adrenalectomy. The data are presented in table 6.

### Discussion

It can readily be seen that the average lymphocyte count per cubic millimeter went down after adrenalectomy in eight animals and rose in seven. The average absolute lymphocyte count was slightly less after operation than before it. A neutrophilic leukocytosis was observed after adrenalectomy.

The results obtained in this laboratory do not agree with the findings of Corey and Britton.<sup>6</sup> The latter workers observed extreme reductions in total leukocyte and neutrophil counts in the adrenalectomized cat. In their animals these alterations resulted in an increase in the per cent of lymphocytes, but the striking blood changes involved the neutrophil. It was suggested that the extreme reductions in total leukocyte and neutrophil counts might be due to *neutrophilogenic* failure resulting from adrenalectomy. The reason for the discrepancy between the findings in this laboratory and those of Corey and Britton are not entirely clear. It is possible that animals used in the earlier work developed infectious feline agranulocytosis<sup>41</sup> during the postoperative period. This highly infectious disease of cats, a condition not known at the time of Corey and Britton's work, would well explain some of the findings.

### Conclusions

In fifteen cats the average total lymphocyte count after bilateral adrenalectomy was not significantly different from the average count prior to operation. A neutrophilic leukocytosis was observed to follow the operative procedure.

## PART II THE PRESENT STATUS OF THE ROLE OF THE ADRENAL CORTEX IN THE REGULATION OF LYMPHOID TISSUE STRUCTURE AND FUNCTION AND OF THE NUMBERS OF CIRCULATING BLOOD LYMPHOCYTES

### A INTRODUCTION

The concept of hormonal control of lymphoid tissue structure and function and of the numbers of blood lymphocytes is an intriguing one. Its most recent and comprehensive evaluation has been made by Dougherty and White.<sup>21</sup> It is the purpose of this review to set forth in a detailed manner the experimental findings which form the foundation for the concept and to attempt to evaluate critically the merits of data both favoring and disfavoring it.

For purposes of convenience the studies relating to hormonal regulation of lymphoid tissue and lymphocytes can be roughly separated into four main groups: (1) Morphologic alterations in lymphoid tissue due to variations in adrenal cortical hormone activity; (2) Effect of varying amounts of available adrenal cortical hormone on the numbers of thoracic duct lymphocytes and rate of flow of thoracic duct



lymph (3) Changes in the numbers of circulating peripheral blood lymphocytes with variations in the available adrenal cortical hormone (4) Alterations in factors thought related to lymphoid tissue and lymphocyte function occurring with varying adrenal cortical hormonal activity The variations in available adrenal cortical hormone have been studied experimentally in many different ways In some instances adrenal cortical extract or purified steroids have been employed to augment the naturally occurring adrenal cortical secretion In other studies the adrenal activity of the experimental subject has been enhanced by the administration of adrenocorticotrophic hormone In still other investigations the subject has been stressed or "alarmed" with the design of calling forth increased adrenal cortical activity as a response to injurious stimuli Conversely, the effects of diminished or absent adrenal cortical hormone have been sought in the experimentally adrenalectomized animal or in human subjects with Addison's disease Most of the evidence has been obtained from animal work However, some evidence has been derived from the effects of hormonal preparations on normal man and from studies on patients with disturbed adrenal cortical metabolism The studies will be discussed under the four groupings mentioned before with the realization that some investigations may overlap into more than one group

## B EVIDENCE OF GROSS AND HISTOLOGIC ALTERATIONS IN LYMPHOID TISSUE ATTRIBUTABLE TO THE ACTION OF ADRENAL CORTICAL HORMONE

### *Review of Medical Literature*

According to Grollman,<sup>30</sup> changes in lymphatic tissue repeatedly have been noted in Addison's disease Clinically these are manifest by enlargement of lymphatic nodules at the base of the tongue, of tonsils, and of other palpable lymphoid tissue Star<sup>65</sup> is credited with having first noted that the thymus in a 17 year old girl dying from Addison's disease was persistent and as large as that of a child twelve months old In addition, in animals dying of chronic adrenal insufficiency there may be, according to Grollman, striking hypertrophy and regeneration of the thymus In young animals subjected to adrenalectomy, the growth of the thymus is stimulated, in older animals, the involuted thymus regenerates This summarization by Grollman is amply supported by the literature both antedating and succeeding his monograph

Joffe,<sup>37</sup> in 1924, using a moderately large group of Wistar rats, clearly demonstrated thymic enlargement in all rats surviving double suprarenalectomy in good condition for from three to five weeks This occurred even though the animal lost weight after the operation Histologically and grossly, the thymus in these animals was uniformly demonstrated to resemble the growing thymus of much younger animals and to differ markedly from the glands of nonoperated controls of the same age The same author<sup>38</sup> reviewed this subject three years later, including the evidence that the suprarenal glands are hypoplastic in patients dying with so-called status thymicolymphaticus Simpson and co-workers,<sup>64</sup> in 1934, found the thymuses of adrenalectomized rats to average 58 per cent greater than those of control animals allowed the same food intake Reinhardt and Holmes<sup>35</sup> likewise found that the thymus and lymph nodes of rats were much heavier in adrenalectomized animals maintained forty-five days on 1 per cent sodium chloride than were those of normal controls or controls receiving sodium chloride but not adrenalectomized

Conversely, the thymus and lymphatic structures have repeatedly been observed to involute following the administration of adrenal cortical hormone or of adrenocorticotrophic hormone to animals with intact adrenals Thus, Moon<sup>48</sup> reported the observation of complete atrophy of the thymus in spayed rats given adrenocorticotrophic hormone This was in marked contrast to the findings in untreated spayed controls Very little objective data was reported Ingle and Mason<sup>36</sup> demonstrated loss of weight of thymic glands in rats in which cortin was implanted subcutaneously in the form of solid pellets Ingle and co-workers<sup>35</sup> administered cortin and purified adrenal cortical preparations by various routes to rats The animals were then killed at intervals and the weight of various organs compared with normal values

The thymus gland showed marked progressive atrophy in those rats treated with cortin in doses of 5 to 10 cc daily. Each cc represented about 75 Gm. of whole adrenal gland. In the same year (1938), Evans et al.<sup>24</sup> reported marked loss of weight of the thymus in rats after administration of adrenocorticotrophic hormone. Ingle<sup>25</sup> demonstrated substantial involution of the thymus in the intact rat after administration of large amounts of cortin and similar atrophy in hypophysectomized rats when additional cortin was supplied animals in which the adrenal cortices had been maintained at normal size by regulated amounts of a renocorticotrophic hormone. Wells and Kendall,<sup>66</sup> in 1940, found the average thymus weight of 6 rats fed large doses of a highly potent, noncrystalline fraction of adrenal cortex was 27 per cent less than that of 20 controls, while the average thymus weight of 6 rats receiving corticosterone was 63 per cent less than that of controls. Neither desoxycorticosterone or its acetate, administered in drinking water or subcutaneously, produced involution of the thymus in these studies. Ingle<sup>34</sup> the same year reported that 2 mg. of 17-hydroxy-11-dehydrocorticosterone administered daily for seven days to 5 adrenalectomized rats lowered the average weight of the thymus to 24.6 mg. from 447.9 mg. in control animals. In a similar study employing the same dose of 11-desoxycorticosterone, there was no significant regression of the thymus in five rats. However, thymic regression of mild degree occurred when the daily dose of desoxycorticosterone was raised to 10 mg. Crede and Moon<sup>8</sup> demonstrated that adrenocorticotrophic hormone produced thymic atrophy in 21 to 23 day old rats when given in three daily injections, and that the degree of atrophy observed was related up to a point to the dose of adrenocorticotrophic hormone. This was also true in control rats but could not be demonstrated in adrenalectomized rats. Noble and Collip<sup>62</sup> were likewise able to confirm the constant presence of thymic atrophy after the administration of corticotrophins to normal and hypophysectomized rats. Dougherty and White,<sup>11</sup> in 1943, showed quite clearly that pituitary adrenocorticotrophic hormone injected in CBA strain mice produced a decrease in weight of the inguinal, axillary and mesenteric lymph nodes and of the thymus. Simpson and co-workers<sup>63</sup> found that the administration of purified adrenocorticotrophic hormone to 6 normal rats resulted in a striking reduction in weight and size of the thymus gland and the cervical lymph nodes as compared to the weight and size of these structures in six untreated controls. This was also readily apparent in 5 treated hypophysectomized animals as compared to 5 untreated hypophysectomized controls, but was not true in 10 adrenalectomized animals when these were compared with untreated adrenalectomized controls. Dougherty and White<sup>14</sup> in an elaborate and intensive study described marked histologic changes in thymic and lymphoid structures of rabbits and mice following administration of adrenocorticotrophic hormone and a variety of adrenal cortical preparations and steroids. The changes did not occur in adrenalectomized animals treated with adrenocorticotrophic hormone nor did they occur in animals given desoxycorticosterone alone. These histologic alterations were grouped by these authors into three stages: (1) The stage of degeneration characterized by pycnosis of medium and small lymphocytes, edema of lymphatic structures, cessation of mitoses and diminished numbers of lymphocytes in nodes. This stage lasted approximately six hours, (2) the stage of repair beginning at six hours and characterized by phagocytosis of debris, and the presence of increased numbers of histiocytes and giant cells, (3) the stage of recovery beginning at about nine hours in mice and characterized by mitoses of remaining lymphocytes, maturation of reticular lymphocytes, and restoration of normal structure.

The occurrence of a similar involution of thymus and lymphoid tissue has long been recognized to follow injurious stimuli known to result in increased adrenal cortical hormone production and hypertrophy of the adrenal cortex. Bardeen,<sup>3</sup> in 1897, observed striking alterations in lymphoid structures throughout the body in autopsy material from patients dying of extensive superficial burns. He commented on the striking similarity of these changes to those produced experimentally in animals by injection of diphtheria toxin, ricin, and other so-called toxalbumins. He also noted similar changes in the lymphatic organs of children dying of diphtheria and other febrile illnesses in which toxemia is thought to be prominent. These findings in burned subjects were confirmed by Pack.<sup>64</sup> Akaiwa and Takashima<sup>2</sup> observed degenerative changes in shielded lymphoid tissue following radiation to other areas. Barnes and Furth<sup>4</sup> made similar observations which they assumed to be due to nonspecific factors carried from damaged tissue by way of the circulatory system. Le Blond and Segal<sup>13</sup> felt that these changes in unexposed lymphatic tissue after radiation were similar to those described by Selye<sup>69</sup> in the alarm reaction after a wide variety of injurious stimuli. They found that this thymic and lymphatic atrophy in unexposed areas was suppressed by adrenalectomy though the general lethal effects of the rays were increased. The observations of Selye<sup>61</sup> and of Foglia and Selye<sup>27</sup> called attention to the fact that injurious

stimuli of many types resulted in thymic involution and hypertrophy of the adrenals, and that none of these stimuli were effective in producing similar changes in the thymus of adrenalectomized animals. Invariable histologic changes were found present in the thymus, spleen and lymph nodes of normal animals after the administration of adrenalin, formaldehyde, morphine, and atropine, and after spinal shock, surgical shock, cold and exercise.<sup>60</sup> Frank<sup>28</sup> confirmed substantial atrophy of the thymus and increase in adrenal weight in rats subjected to spinal cord transection. Zeckwer<sup>74</sup> found that loss of weight of thymus and lymph nodes resulted in rats injected subcutaneously with formalin irrespective of previous castration, thyroidectomy or thyroid feeding. Dougherty and White<sup>18, 71</sup> showed that lymphoid tissue involution produced by inanition was mediated by the adrenal cortex. The evidence that a variety of injurious stimuli cause involution of lymphoid and thymic tissue and that this is dependent upon an intact hypophyseo-adrenal system is more elaborately summarized by Selye<sup>59</sup> in a recent review. Suffice it to say here that the evidence is extensive, well documented and that there is little in the way of dissenting studies.

### *Summary and discussion*

It appears reasonable therefore to regard as proved that adrenal cortical hormone is capable of producing gross and histologic changes of an involutionary nature in lymphoid and thymic structures. The weight of protocols supporting this thesis, the lack of conflicting evidence, and the almost complete unanimity of opinion in this regard leave little doubt as to its validity. It can probably be safely assumed that augmentation of adrenal cortical hormone (more particularly the sugar controlling hormone) is reflected by loss of weight of lymphatic structures and the thymus, that diminution in the amount of this hormone elaborated results in increase in weight and hyperplasia of these structures, and that involutionary alterations can be produced in the intact but not the adrenalectomized animal by appropriate injurious stimuli.

The translation of these well established observations into terms of lymphoid tissue function is more difficult. That such structural changes occur is undoubted. Why they occur and just what they mean are more abstruse questions. It has been suggested<sup>14</sup> that these structural changes represent a mechanism for liberating the metabolically important contents of lymphocytes in increased amounts during a period of stress. Increased rate of dissolution of lymphoid tissue has been assumed to contribute to the normal defense of the stressed organism in at least three ways:<sup>14</sup> (1) Increased release of globulin from lymphocytes—possibly providing precursors for carbohydrate synthesis, (2) release of antibody globulin in the immunized animal, (3) increased production of macrophages in lymphoid structures. However, the relationship of structural changes in lymphoid tissue to other events resulting from variations in adrenal cortical activity is of necessity one of inference, and final proof must rest on the conclusive demonstration that a cause and effect relationship exists between morphologic alterations in lymphoid structures and other changes attributable to adrenal cortical hormone.

### C EVIDENCE FOR ALTERATIONS IN THE NUMBERS OF THORACIC DUCT LYMPHOCYTES IN ANIMALS AFTER ADMINISTRATION OF ADRENAL CORTICAL HORMONE OR ADRENOCORTICOTROPIC HORMONE

The evidence for hormonal effects on the numbers of thoracic duct lymphocytes<sup>15</sup>, to the authors' knowledge, confined to three investigations. One of these is the study reported in Part I of this paper. The subject has been discussed in detail in

the experimental section of this report and it would be redundant to again review it here. Suffice it to say that an effect of adrenal cortical hormone on the numbers of thoracic duct lymphocytes or flow of thoracic duct lymph cannot be regarded as proved. The studies in this laboratory failed to demonstrate any significant effect of adrenal cortical extract on the thoracic duct lymphocyte in contrast to the earlier reports suggesting such effects exist.

#### D EVIDENCE FOR ALTERATIONS IN THE NUMBERS OF BLOOD LYMPHOCYTES ATTRIBUTABLE TO AUGMENTATION OF AND DIMINUTION IN THE AMOUNT OF AVAILABLE ADRENAL CORTICAL HORMONE

##### *Review of Medical Literature*

Evidence for adrenal cortical control of the level of circulating blood lymphocytes takes origin from many sources. Most of the experimental work reported has been in mice and rats, a little in rabbits, a very little in man.

Recently De LaBalze, Reifstein, and Albright<sup>10</sup> analysed the blood counts of 10 patients with Cushing's Disease, 20 patients with Addison's disease, and 20 patients with panhypopituitarism. Total and differential leukocyte counts were done by the hospital staff or laboratory technicians. The average white blood cell count and average absolute neutrophil count were found to be high in Cushing's disease, while the absolute lymphocyte count was low (1500 per cu. mm.) but within the accepted normal range. In all other groups the average figures for these factors were within the normal range. Population studies indicated that the patients with Addison's disease tended to have lower leukocyte and absolute neutrophil counts than patients with Cushing's disease. The *relative* per cent of lymphocytes was below 20 in all cases of Cushing's disease, was 25 to 35 in 8 cases of Addison's disease and over 35 per cent in 11 cases. It is well to point out that the 20 cases of Addison's disease could most certainly not be separated from *normal* individuals on the basis of the numbers of circulating lymphocytes, that while the absolute lymphocyte counts in Cushing's disease showed a tendency to be low these patients had concomitant leukocytosis and neutrophilia, and that by far the most striking difference between the two conditions was found in the average absolute neutrophil counts (4403 per cu. mm. in Addison's disease, 9299 per cu. mm. in Cushing's disease).

In addition to these human studies relating to the blood lymphocytes in disturbances of adrenal cortical function, a number of observations have been made in experimentally adrenalectomized animals. In 1928, Zwemer and Lyons<sup>75</sup> observed a decrease in polymorphonuclear leukocytes and an increase in the per cent of small lymphocytes in 7 cats surviving bilateral adrenalectomy for more than four days. This relative shift in leukocyte distribution did not occur till about the fourth day, leukocytosis with neutrophilia being the rule before that time—particularly in animals surviving less than four days. In 1932, Corey and Britton<sup>6</sup> suggested a *neutrophilogenic* failure to account for extreme reductions in total leukocyte and neutrophil counts in the adrenalectomized cat. In one animal the relative neutrophil count fell to 3 per cent. These alterations of course resulted in an increase in the per cent of lymphocytes, but the striking blood changes involved the neutrophil. Data from this laboratory on blood counts of cats before and after adrenalectomy are presented in Part I of this paper. They did not confirm the earlier work and failed to demonstrate significant changes in absolute lymphocytes in the cat after adrenalectomy. The discrepancy between this and the earlier work is not explained but it is suggested that possibly the occurrence in the latter of infectious feline agranulocytosis (a condition not known at the time of Corey and Britton's work) in animals during the postoperative period could result in the blood picture found. Simpson and co-workers,<sup>64</sup> in 1934, published a few observations on blood counts of adrenalectomized rats. They found no significant change from controls in either total leukocyte count or per cent lymphocytes in animals suffering from chronic adrenal insufficiency. However, counts were made on only a few controls and adrenalectomized rats. The following year, Shecker, Friedman and Nice<sup>67</sup> published a short summary of mean values for leukocytes in the blood of adrenalectomized animals. In 34 normal rats the mean leukocyte count per cu. mm. was 11,319, whereas it had risen to 17,576 per cu. mm. just before death. In 19 rats differential leukocyte counts showed 81.2 per cent lymphocytes before adrenalectomy.

and 88.4 per cent lymphocytes immediately before death from adrenal insufficiency. The figures given compare the normal with the moribund rat. In 1940, Dalton and Masson<sup>9</sup> reported data on 8 rats comparing the preoperative count with counts made twenty-four hours before death from adrenal insufficiency. There was an absolute lymphocytosis, an absolute neutrophilia, and a marked erythrocytosis, suggesting a severe degree of hemoconcentration in each case. It is again unfortunate that the comparison was made for animals in the terminal stages of adrenal insufficiency. More recently, White and Dougherty<sup>67</sup> have reported blood findings in adrenalectomized rats and mice. In 14 adrenalectomized mice, the lymphocyte counts per cu mm were  $14,549 \pm 1514$  (means and standard errors) as compared to an average of  $9584 \pm 371$  in 99 normal controls. Since these animals were untreated, hemoconcentration undoubtedly occurred. However, in adrenalectomized mice partially controlled with desoxycorticosterone the average lymphocyte count was  $11,674 \pm 684$ . The data for mice were obtained in the first five days post adrenalectomy. In the figures presented for rats no change in absolute lymphocyte count was noted until six days after adrenalectomy. Crafts<sup>7</sup> was unable to demonstrate any significant change in total or differential leukocyte counts in adrenalectomized rats maintained on 1 per cent sodium chloride.

Since 1943, there have been a number of studies of the effects of adrenal cortical hormone on the blood elements. Dougherty and White,<sup>17</sup> in 1943, found leukopenia, lymphopenia, and neutrophilia in mice administered adrenocorticotrophic hormone. This was apparent in three hours and change was maximal in nine hours after hormone administration. Similar results were obtained when adrenal cortical extract was given except that these findings occurred earlier. No such alterations resulted when adrenocorticotrophic hormone was given to adrenalectomized animals. In 25 mice receiving daily adrenocorticotrophic hormone for fifteen days, there was lymphopenia, neutrophilia, and little change in total leukocyte count. The blood picture could not be similarly altered by injection of prolactin, pitressin or serum globulin.

The following year, Reinhardt and co-workers<sup>67</sup> reported that two injections of 1 mg of adrenocorticotrophic hormone four hours apart resulted in lymphopenia and an increase in neutrophils in 6 rats as compared with 6 controls. In 4 dogs given adrenocorticotrophic hormone intraperitoneally, the marked leukocytosis developing and the differential cell counts obtained were not significantly different from controls given other protein substances by the same route. However, 3 dogs given 10 mg of adrenocorticotrophic hormones subcutaneously showed leukocytosis and some reduction in blood lymphocytes after six hours. Two controls showed little change in absolute lymphocytes. The controls also did not develop leukocytosis, however.

The most intensive studies of the role of pituitary adrenocorticotrophic hormone in regulating the numbers of blood elements have been made by Dougherty and White.<sup>13</sup> Observations were reported in mice, rats, rabbits and a few in man. In mice single injections of adrenocorticotrophic hormone were given, groups of animals bled at intervals after injection, and the average cell counts compared with controls. Results indicated a substantial lymphopenia developed within one to three hours. This attained a maximum at nine hours and returned to normal by approximately twenty-four hours. In rats similarly studied, absolute lymphopenia and leukopenia were apparent at three, six and nine hours after injection and these were still present up to twenty-four hours. In rabbits, considerable lymphopenia was found up till twenty-four hours after injection. Desoxycorticosterone did not produce similar changes. In most instances, an increase in granular leukocytes accompanied the lymphopenia. Adrenocorticotrophic hormone was reported to cause neutrophilia but not lymphopenia in adrenalectomized animals. It was also stated that other protein substances will produce leukocytosis but not lymphopenia. In the same groups of studies, lymphopenia was observed in mice, rats, and rabbits receiving a single dose of 0.5 cc, 2.0 cc, and 5 cc of adrenal cortical extract respectively. In 5 human cases not suffering from Addison's disease, 20 cc of aqueous adrenal cortical extract were administered subcutaneously and blood counts made three and six hours afterwards. The results were equivocal, showing slight reduction in absolute lymphocyte counts after injection. However, the data must be viewed with some question since erythrocyte counts in the six hour period following hormone injection showed extreme unphysiologic variations in 2 of the 5 cases. Counts in both of these individuals varied from less than four to over five million per cu mm without similar changes in the hemoglobin values.

Elmadjian and Pincus<sup>25</sup> obtained blood lymphocyte values at intervals throughout the day on a small group of normal and psychotic patients. A regular diurnal rhythm was reported in normal but not in psychotic subjects. This was said to correlate with 17 ketosteroid output in urine, the latter being greatest when the lymphocyte counts were lowest. An irregular ketosteroid pattern was observed in psychotics.

Yoffey and Baxter<sup>22</sup> in 1946 found a reduction in absolute lymphocyte counts in rats after injection of adrenocorticotrophic hormone or adrenal cortical extract. No mention was made of the total leukocyte values in this group. In 8 rats receiving daily injections of adrenocorticotrophic hormone, adrenal cortical hormone, or both for twenty-eight days, the lymphocyte count twenty-four hours after the last dose was elevated in 3 animals and depressed in 5. The significance of counts made as long as twenty-four hours after the last injection is, of course, open to question. In 2 rabbits subjected to a twenty-two day control period followed by daily subcutaneous injection of adrenal cortical extract, no appreciable change in absolute lymphocyte count was obtained.

Very recently, a detailed study at the Mayo Clinic<sup>46</sup> has been reported on the results of the administration of anterior pituitary adrenocorticotrophic hormone to a young woman maintained on a constant diet. The total daily dose was 25 mg. for six days, 50 mg. for six days, and 100 mg. for eleven and one-half days. Changes in lymphocyte count were regarded as of questionable significance and the plasma electrophoretic pattern remained unchanged during the entire period of study.

Harlow and Selye,<sup>31</sup> in 1937, reported leukocytosis with relative and often absolute lymphopenia in rats and mice given adrenalin or formaldehyde or exposed to cold or surgical shock. The changes in the blood picture during the alarm reaction which has been demonstrated to elicit increased adrenal cortical activity have been recently summarized by Selye.<sup>69</sup> The occurrence of neutrophilic leukocytosis with accompanying lymphopenia was emphasized.

A few studies are available on the effects of adrenal cortical activity on leukemia in animals. Murphy and Sturm,<sup>49</sup> in 1943, observed that adrenalectomy substantially increased the percentage of cures of a transmissible leukemia in rats. The same authors<sup>50</sup> found increased survival when rats of highly susceptible strain were treated with adrenal cortical preparations following intraperitoneal inoculation of leukemic cells. Law and Speirs<sup>40</sup> noted a decrease in total lymphocytes and in the immature lymphocytes of mice with spontaneous lymphoid leukemias after intraperitoneal or subcutaneous injection of adrenal cortical extract (aqueous) or lypoadrenal extract. Regression of infiltrated thymuses and lymph nodes and to a lesser extent of the spleen was observed. In the small series of animals studied, the effect on life expectancy could not be stated.

### *Summary and Discussion*

There is considerable evidence to suggest that in mice and rats the administration of appropriate amounts of adrenocorticotrophic hormone, adrenal cortical hormone, or the stimulation of the adrenal gland by "alarming" the animal results in the production of some degree of absolute lymphopenia. There is somewhat less evidence that this applies to the rabbit, only equivocal evidence that it applies in the dog, and scant evidence thus far for its application to man. Recently, Dougherty and White<sup>21</sup> have reported receiving personal communications to the effect that changes of this nature have been observed in man. In most instances, the lymphopenia has been accompanied by neutrophilia. More work is needed on the nature of the frequently reciprocal behaviour of the granular leukocyte and the lymphocyte. It is perhaps unfortunate from the standpoint of critical review that so many of these studies have been conducted on the mouse and rat, animals in which the lymphocytes constituted 60 to 90 per cent of the blood leukocytes in contrast to certain other mammals, including man, in which the neutrophil/lymphocyte ratios is the reverse of this. These experimental animals, because of their size and ready availability, are admirably suited for group hematologic studies. However, the differences in blood picture from that seen in man raise the question as to how literally observations in these animals can be applied to man. Be that as it may, in summarization it is fair to state that there is some evidence that absolute lymphopenia does result after augmentation of adrenal cortical hormone.

Conversely, the evidence for lymphocytosis resulting from diminution in the amount of available adrenal cortical hormone is at present none too satisfactory. Observations in adrenalectomized animals have been scanty and frequently open to the criticism that comparisons were made between normal animals and animals in which severe derangement of many types had occurred with extreme symptoms of adrenal insufficiency. Further, not all observers have reported absolute lymphocytosis and lymphocytosis, when observed, has not always been of striking nature. It is not clear that the absolute lymphocyte counts of patients with Addison's disease differ from those of normal individuals. It is the opinion of the authors that the present available evidence admits some reservation that the regulation of the numbers of blood lymphocytes is under pituitary control and mediated by way of the adrenal cortex.

#### EVIDENCE OF ALTERATIONS IN FACTORS ASSOCIATED OR PRESUMED ASSOCIATED WITH LYMPHOCYTE AND LYMPHOID TISSUE FUNCTION ATTRIBUTABLE TO AUGMENTATION OF OR DIMINUTION IN THE AMOUNT OF AVAILABLE ADRENAL CORTICAL HORMONE

##### *Review of Medical Literature*

The functions of the lymphocyte have been the subject of much speculation and discussion. Drinker and Yoffey<sup>22</sup> summarized the available information up to the time of the publication of their monograph (1941) and were inclined to agree with Lewis that while the functions of lymphocytes were the subject of religious beliefs, reliable factual information as to their specific functions was sparse. However, lymphoid tissue has long been suspected of playing a role in serologic immunity. McMaster and Hudack<sup>47</sup> summarized the literature in this regard in 1935, presenting also evidence of their own that agglutinins were formed within the draining lymph nodes of mice following intradermal injection of killed cultures of micro-organisms. These authors were careful to point out that their experiments threw no light upon the subject of antibody formation elsewhere in the body. They felt that there is no good reason to consider the lymph nodes as the sole site of antibody formation. The subject was again reviewed by Drinker and Yoffey in their monograph.<sup>22</sup>

Since 1940, further evidence has accumulated to support the suspicion that lymphoid tissue and lymphocytes may be one of the factors concerned with the production of serum globulin and antibodies. In the study of Ehrlich and Harris,<sup>23</sup> antigens were injected into the footpad of rabbits and the subsequent formation of antibodies in the single popliteal node draining the area was compared with the output of lymphocytes in the efferent lymph from this node and with morphologic changes in the node. Antibodies were found to appear in the efferent lymph two to four days after injecting antigen and reached their height after six days. The rise in serum titer was preceded by a sharp rise in the output of lymphocytes in the efferent lymph and by hyperplasia of the node. The authors felt the evidence pointed to the lymphocyte as a factor in the formation of antibodies.

Dougherty, Chase and White,<sup>16</sup> two years later, extracted the washed cells from minced pooled lymphoid tissues of immunized and nonimmunized mice and also made extracts of nonlymphoid tissues. Extracts of lymphoid tissue from immunized mice showed high titers of antibodies. No titer was found in extracts from salivary glands or muscles of immunized animals nor from lymphoid tissues of nonimmunized animals. The authors concluded that antibodies are concentrated in lymphocytes. The actual production of antibodies by lymphocytes has not been established.

Kass,<sup>39</sup> in 1945 studied the thoroughly washed lymphocytes from lymph nodes removed from a patient one hour after death. Although the cell suspension of lymphocytes was washed until the supernatant would not react with rabbit antisera prepared using highly purified human gamma globulin as antigen, the extract of the lysed cells themselves reacted to anti-human globulin rabbit sera. When mixed with antisera, this extract inhibited the ability of the antisera to precipitate human gamma globulin or serum.

In the same year, Harris and co workers<sup>37</sup> observed that cell extracts from the popliteal node draining a site of antibody formation in the rabbit contained significantly higher titers than efferent lymph plasma. The greatest difference between cells and lymph plasma was found five days after the injection of antigen. These results were interpreted to indicate either (1) lymphocytes are a source of antibody formation or (2) lymphocytes absorb or adsorb antibody. Evidence was presented favoring the first view since lymphocytes both *in vivo* and *in vitro* were demonstrated not to pick up antibodies with which they were placed in contact.

With this as background, efforts have been made to correlate serum globulin and antibody titer changes with the postulate of the adrenal cortical control of lymphoid tissue and the blood lymphocyte level. White and Dougherty<sup>69</sup> found a slight but statistically significant rise in serum proteins in rats three and six hours after administration of adrenocorticotrophic hormone. This was back to normal within twenty-four hours. Daily administration of adrenocorticotrophic hormone for fifteen days produced serum protein levels somewhat above that of controls. It was thought that lymphocytes were the source of the serum protein rise. The same authors<sup>68</sup> in a more extensive report obtained a statistically significant increase in total serum protein of rabbits in the twenty-four hour period following administration of 10 mg of adrenocorticotrophic hormone or 5 mg of adrenal cortical steroids in oil. This was found due to an increase in the beta and gamma globulins. Cell extracts of lymphoid tissue were also found to have protein components identical with gamma globulin of serum. The authors concluded that this evidence in conjunction with observation on the lymphoid tissues and blood lymphocytes justifies the assumption that the rate of release of protein from lymphocytes is under the normal physiologic control of the pituitary adrenocorticotrophic hormone which exerts its influence by way of the adrenal cortex.

Dougherty, White and Chase,<sup>12</sup> in 1944, reported an increase in antibody titer after a single injection of adrenal cortical extract in 8 rabbits whose titer could not be further elevated, by injections of antigen alone. In rabbits immunized with sheep erythrocytes final agglutinin titers were higher if adrenal cortical extract was administered together with the antigen.

The same workers<sup>16</sup> produced an anamnestic reaction in rabbits and mice following a single injection of adrenal cortical extract or adrenocorticotrophic hormone. Toxic stimuli such as benzene and potassium arsenite, produced an anamnestic response in normal but not in adrenalectomized mice. It was concluded that the data established the role of pituitary-adrenal secretion as the controlling mechanism for the release of antibody from lymphocytes. The anamnestic reaction is a manifestation of this control. The following year, Dougherty, White and Chase<sup>19</sup> found that lymphocytes obtained by extraction of cells from rapidly growing mouse lymphosarcoma in immunized animals contained antibody. The antibody-containing tumor could be transplanted into a second generation of mice and antibody was subsequently found in the normal lymphocytes of the animal in which the transplant was made. It is interesting that antibody was found in the presumably normal lymphocytes of the animals who had never been exposed themselves to antigen.

Chase, White and Dougherty,<sup>6</sup> in 1946, presented evidence that in mice, rats and rabbits antibody production reached a higher maximal level when adrenal cortical extract was injected with antigen. In rabbits hyperimmunized to sheep erythrocytes, a single subcutaneous injection of adrenal cortical extract produced an increase in antibody titer. The elevation in titer in most experiments was of the order of one tube when converted to terms of the usual double dilution technic. However, the trend in all experiments appeared to be uniformly upward. Continued daily injection of adrenal cortical steroids in oil were reported to maintain higher titers than those seen before hormone administration. Eisen and co workers,<sup>41</sup> on the other hand, found no difference in either antibody titers (sheep cell agglutinins and hemolysins and precipitins against pneumococcus capsular polysaccharide or serum gamma globulin) in adrenalectomized rats receiving adrenal cortical liporextract from similar animals receiving only desoxycorticosterone. Studies with glycine tagged with heavy nitrogen showed no differences in the rate of nitrogen metabolism in serum proteins in the two groups.

The effect of roentgen radiation, known to be highly destructive to lymphoid tissue, has also been investigated in connection with hormonal control of release of protein and antibody from lymphoid tissue.<sup>71, 72</sup> Whole body radiation in the amount of 200 r was found to elevate total serum proteins and gamma globulin and to produce an anamnestic response in previously immunized adrenalectomized mice.



The much smaller dose of 10r produced the same physiologic alterations including the anamnestic response in normal but not in adrenalectomized mice. It was felt by the authors that small doses of radiation exert their action on lymphoid tissue by augmenting pituitary-adrenal cortical secretion while large doses produce their effect without endocrine mediation.

Recently, Gjessing and Chanutin<sup>29</sup> have reported the results of electrophoretic analysis of plasma and plasma fractions of normal rats and rats subjected to thermal injury. The injured rats exhibited a decrease in the per cent of gamma globulin and albumen fractions. Alpha and beta globulins were increased. These authors report that their unpublished observations indicate that similar changes occur in the dog and that these injury patterns of Fraction II and III were also observed after administration of terpineol or of adrenal cortical hormone. In other studies involving the Tiselius apparatus, Lewis and McCulloch<sup>44</sup> found a decrease in gamma globulin and albumen in 8 cases of Cushing's syndrome. Li and Reinhardt<sup>45</sup> could detect no increase in the gamma globulin fraction of plasma of either hypophysectomized or normal rats administered adrenocorticotrophic hormone. The albumen fraction was increased. The protein components of cervical duct lymph in these animals was not altered by adrenocorticotrophic hormone. Nitske and Cohen,<sup>61</sup> employing methanol fractionation, were unable to demonstrate statistically significant alterations in the serum proteins of a group of 12 patients with lymphocytic leukemia.

### *Summary and Discussion*

There is rather strongly suggestive evidence that lymphocytes or lymphoid tissue may be a source of gamma globulin and play a role in antibody formation. There is some evidence that in certain species antibody titers may be enhanced and that anamnestic responses of considerable magnitude may be elicited by the administration of adrenal cortical hormone. The demonstration of significant changes in plasma gamma globulins after hormone administration requires further confirmation. Increases in gamma globulin reported have been relatively small and other observers have found decrease in gamma globulins after stress<sup>29</sup> or no changes after administration of adrenocorticotrophic hormone.<sup>46</sup> Further, some investigators<sup>23,47</sup> have found that antibody production is accompanied by histologic hyperplasia of lymphoid tissue rather than by involutionary changes, and impairment of antibody production in the adrenalectomized animal has not been consistently demonstrated. Joffe<sup>38</sup> and Grollman<sup>30</sup> review evidence that, if anything, antibody production in adrenalectomized animals is increased. Chase, Dougherty, and White<sup>5</sup> point out that the matter is controversial.

The hormones of the adrenal cortex have manifold metabolic activities. It is difficult at the present time to regard as proved that the reported changes in plasma globulins and antibody titers necessarily have resulted from lymphocyte dissolution or that the release of antibody is normally under adrenal cortical control. Further work will have to be done before this important point can be settled.

### GENERAL SUMMARY AND DISCUSSION

The hormonal control through the hypophyseo-adrenal cortical system of lymphoid tissue structure and function is an important concept. We cannot at the present time regard that the concept is established fact. Final judgment must await additional work and the clarification of some of the inconsistencies which appear to exist. It seems reasonable that lymphoid tissue is one of the end organs of adrenal cortical hormone and that it may perhaps play a role in the response of the organism to stress. It seems quite clear that the sugar hormone of the adrenal cortex is capable of producing structural alterations in lymphoid tissue. Change

in thoracic duct lymphocyte numbers as a result of augmentation in the amount of available adrenal cortical hormone is at present controversial. Experiments in this laboratory have failed to demonstrate it. The production of lymphopenia, at least in some species and possibly in man, by increasing available sugar hormone is supported by some evidence. The exact mechanism of production of lymphopenia is open to question, its relationship to changes in lymphoid tissue structure being one of inference. The converse situation—absolute lymphocytosis resulting from deprivation of adrenal cortical hormone—is the subject of controversial reports. At best, it must be admitted that relatively slight alterations from the accepted normal range of lymphocyte values occur in the adrenal insufficient organism. Changes in plasma gamma globulins and antibody titers associated with changes in the amount of available cortical hormone are reported. It should be clarified whether such changes have necessarily resulted from lymphocyte dissolution or are related to other of the variegated actions of adrenal cortical hormone.

The relationship of adrenal cortical hormone to lymphoid tissue and lymphocytes and the relationship of the latter to the response of the organism to stress must indeed be complex. It is reasonably well established that the life span of the lymphocyte is very short indeed<sup>1 58 22</sup> and each lymphocyte presumably liberates its metabolically important contents within a few hours at the most. If stress continues for any period of time, as often it does, it is difficult to visualize the wisdom of interfering with the production of metabolically vital substances in order to secure the transient benefits of lymphoid tissue dissolution. It is also somewhat difficult to regard as proved that the various changes reported after hormone augmentation or deprivation necessarily represent the *normal* mechanism by which these factors are regulated and kept within physiologic limits. More investigations are required to answer such questions and to further elucidate the interrelationship of the adrenal cortex and lymphoid tissues.

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# CHANGES IN CIRCULATING LEUKOCYTES INDUCED BY THE ADMINISTRATION OF PITUITARY ADRENOCORTICOTROPHIC HORMONE (ACTH) IN MAN

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CLEMENT A FINCH, M D

## INTRODUCTION

IN THIS communication we report observations on the hematologic effects of adrenal stimulation by pituitary adrenocorticotrophic hormone (ACTH). Experiments by other investigators<sup>1 2 3</sup> have demonstrated that circulating leukocytes in animals are subject to the influence of the adrenal cortex. The aim of this study has been to clarify, by direct stimulation of the adrenal cortex, the nature of the control exerted upon the leukocytic pattern by adrenal cortical secretion in man, and in this way to throw some light upon the mechanism of leukocyte alterations in Addison's disease, as well as in conditions characterized by adrenal overactivity.

## METHODS

The preparation\* employed in this study was a purified adrenocorticotrophic hormone derived from hog pituitary by isoelectric precipitation in the cold. Its potency is attested by the fact that intravenous administration of 2 to 4 micrograms into a hypophysectomized rat provoked a marked decrease in the ascorbic acid content of the adrenals. Electrophoretic analysis revealed the presence of more than one component. The material had 0.12 units of oxytocic activity and 0.1-0.2 pressure unit per milligram, growth promoting, gonadotropic and thyrotropic factors were not present in significant amounts. It is a white amorphous powder which is soluble to at least 5 mg per milliliter of saline, although some lots require either slight alkalization or acidification for complete solution. It was handled with sterile precautions and dissolved in sterile media. No attempt was made to sterilize the material itself, as ultrafiltration results in considerable loss of biologic activity.

Smears were made directly from the needle of the syringe and prepared with Wright's stain. All other hematologic studies were carried out on oxalated venous blood. Absolute neutrophil and lymphocyte counts were calculated from the total leukocyte count and the smear differential.

Eosinophils were enumerated directly according to the method of Dunger,<sup>4</sup> slightly modified. By this technic, oxalated blood is drawn into an ordinary white cell pipet to the 0.5 mark. A special diluting fluid is used, consisting of 5 cc of 2 per cent aqueous eosin, 5 cc acetone and 90 cc distilled water. The hypotonicity of the solution causes the red cells to rupture, the granules of the eosinophils take up the red dye while other leukocytes remain colorless. A Levey hemocytometer

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\* This material was kindly supplied through Dr. John R. Mote of the Armour Laboratories, Chicago, Illinois.

(depth  $\frac{2}{10}$  mm) is used and 4 chambers of 16 sq mm each are counted. Under these conditions, one counts, with normal human blood, about 10 to 35 cells in each chamber. The eosinophil count per sq mm (normal range—60 to 200) is obtained by multiplication of the average of 4 chamber counts by a factor of 6.25. The method permits a high degree of accuracy.<sup>5</sup> Should it be desirable, the counting sensitivity can be doubled by merely drawing blood to the 10 mark in the leukocyte pipet.

Two technical points may be stressed. First, the pipet should be shaken only 50 to 150 times and the chamber loaded promptly, since Dungen's solution tends to rupture leukocyte membranes. Second, after the fluid has been introduced into the chamber, two to three minutes should be allowed to elapse while the granules

TABLE 1-a—Effect of ACTH on Leukocytes—Normal Volunteers

Patient, Age, Sex, PBBH Hosp. No.	8 A.M.				12 Noon				% Change		
	WBC	Neut	Lym	Eos	WBC	Neut	Lym	Eos	Neut	Lym	Eos
M. K., 26, F, No. 5A491	5700	2793	2508	97	10400	8112	1872	19	+191	-76	-80
C. L., 27, F, No. 5A492	5800	3248	2262	88	9700	8536	970	19	+163	-57	-78
L. D., 27, F, No. 5A493	6900	3795	2691	79	7500	5550	1575	2	+46	-42	-97
J. C., 29, F, No. 5A494	7000	4410	2590	81	10800	8316	2160	9	+89	-17	-89
V. P., 24, M, No. 5A295	7500	3975	3225	212	9266	6946	2162	78	+75	-33	-63
W. H., 22, M, No. 5A296	10000	6000	3600	344	12735	10805	1660	100	+80	-54	-71
P. L., 26, M, No. 5A343	12300	7995	4000	288	12790	10418	2098	61	+31	-48	-79
R. P., 28, M, No. 5A297	7750	4620	2850	169	11150	9554	1838	61	+107	-36	-64
Average									+98	-45	-75

take up the dye. When this is done, no difficulty will be encountered in identifying the darkly stained eosinophils at a glance.

## RESULTS

### I. EFFECT OF A SINGLE INJECTION OF ACTH

#### A. Effect in Subjects without Adrenal Cortical Impairment

The effect of a single intramuscular injection of 25 mg of ACTH, given at 8 A.M., was studied in fasting human subjects. After an initial short latent period, there ensues a fall in lymphocytes and eosinophils and a rise of neutrophils, commonly resulting in an increase of the total leukocyte count. These changes are always apparent by two hours after injection, and become maximal at about four hours. There then ensues a return of the leukocytes over the next four to six hours to levels approaching the pre-injection ones. Determinations of the effect of a given dose of hormone after four hours thus represents the maximum measurable change. Significant changes are not observed under identical control conditions without hormone administration.

Determinations of the changes in circulating leukocytes found four hours after injection of 25 mg of ACTH intramuscularly in 8 normal human volunteers and in 38 patients with miscellaneous conditions are recorded in tables 1-a and 1-b. The

TABLE 1b - Effect of ACTH on Leukocytes in Miscellaneous Conditions

Patient	Age	Sex	PBBH Ho p No	Diagnosis	8 AM				12 Noon				% Change		
					WBC	Neut	Lym	% Eos	WBC	Neut	Lym	Eos	Neut	Lym	Eos
1	J B, 59	M	No 71545	Terminal haemorrhagic cirrhosis				54				19			-65
2	D B, 10	M	No 1A460	Rheumatoid arthritis				97				19			-80
3	D B, 34	F	No 5A762	Disseminated lupus	2400	1540	697	36	4500	3860	540	1	+87	-23	-98
4	H C, 24	M	No 6A680	Thyrotoxicosis (controlled on iodine)	8900	4700	3470	185	10875	8800	1520	32	+87	-56	-82
5	A C, 33	F	No R3703	Thyrotoxicosis (uncontrolled)	4500	2400	1620	410	6100	4500	1470	210	+88	-9	-49
6	D L, F*			Myasthenia gravis	9050	5250	3080	136	10900	9380	1530	52	+78	-50	-62
7	M F, M*			Myasthenia gravis (thymectomy)	11250	6980	3830	384	14600	12550	2040	119	+80	-40	-69
8	M F, 19	F	(RBBH)**	Rheumatoid arthritis				262				37			-86
9	W M, 13	M	No R6612	Osteoporosis	7550	4075	3395	128	13650	11200	2595	51	+175	-24	-60
10	J G, 67	M	No F7789	Gastric and duodenal ulcers	2800	1960	672	125	3650	3390	256	19	+73	-62	-85
11	M G, 29	F	No M5291	Neurasthenia				234				88			-62
12	F McG, 54	M	No 6A346	Bronchial asthma	9300	4460	3070	405	11150	7700	2008	187	+73	-35	-53
13	J M, 45	F	No 6A913	Rheumatic heart disease	7500	3980	3302	36	7400	5250	1850	13	+24	-44	-64
14	C N, 62	F	No 5A417	Familial hemolytic jaundice (splenectomy)	6650	2530	3460	117	8600	4760	2580	8	+128	-25	-90
15	L P, 45	F	No 16265	Anxiety state				200				97			-52
16	J R, 41	F	No D8627	Cushing's syndrome	9785			23	12000			9			-61
17	C S, 36	M	No 2A64	Rheumatic fever				216				50			-77
18	N S, 25	M	No 5A675	Old poliomyelitis	7400	4695	2510	231	10600	9200	1170	69	+97	-53	-70
19	E S, 47	F	No 6A444	Pituitary hypothyroidism				144				50			-65
20	M S, 23	F	No 6A848	Hypothyroidism	5400	3080	2050	58	9200	7640	1288	17	+148	-37	-71
21	E L, 45	F	No 5A86	Chronic brucellosis	12450	8500	2860	286	17350	15800	1380	25	+86	-52	-91
22	G G, 16	F	No 5A821	Psychoneurosis	5300	2915	2279	210	7850	5662	2189	42	+94	-4	-81



TABLE 1-b—Concluded

Patient Age Sex, PBBH Hosp No	Diagnosis	8 A.M.				12 Noon				% Change		
		WBC	Neut	Lym	10 <sup>3</sup>	WBC	Neut	Lym	10 <sup>3</sup>	Neut	Lym	Eos
23 W H, 23, M, No 5A796	Grippe				156				13			-80
24 M H, 25, F, (MGH)*	Myasthenia gravis				188				23			-88
25 G H, 51, M (MGH)*	Myasthenia gravis				56				8			-86
26 F H, 41, M, No 5A994	Reactive hypogly- cemia	6100			108	12000			33			-70
27 L H, 14, M, No 5A605	Obesity ? Froe- lich's	5600	2520	2410	280	10700	8800	1500	76	+248	-38	-72
28 J H, 24, M, No 5A985	Anorexia	6000	2700	2580	239	8550	6840	1370	69	+153	-47	-71
29 I K, 43, F, No D8695	Scleroderma	9100	6900	2000	210	15005	13800	900	59	+100	-55	-72
30 R L, 53, M, No R6318	Thyrototoxicosis (poorly con- trolled)				202				52			-74
31 G L, 50, F, No 6A92	Chronic hepatic insufficiency				99				17			-83
32 D McA, 19, M, No M8487	Pituitary hypo- gonadism	7000			47	10000			12			-75
33 A C, 44, M, No 5A772	Asthenia	7700	4850	2460	175	12850	11450	1412	48	+136	-43	-73
34 A D, 19, F, No 5A459	Epilepsy	11500	5180	5400	194	10100	7440	2220	69	+44	-59	-65
35 A D, 52, F, No 5A500	Allergic rhinitis	8100	4290	1860	780	13600	11550	1360	206	+170	-27	-74
36 W P, 19, M, No 5A151	Primary syphilis	10950	6899	4055		14500	12325	2175		+78	-46	
37 A S, 48, F, No 5A713	Chronic brucello- sis	14775	10850	3250	259	13650	10650	3000	112	-2	-8	-57
38 M P, 52, F, No 1A436	Thyrototoxicosis	5400	2110	2865	125	6450	5030	1290	3	+138	-55	-93
Average										+104	-39	-73
Grand Average (Tables 1-a and b)										+102	-40	-73

\*We were permitted to study these patients through the courtesy of Dr Henry R Viets of the Massachusetts General Hospital, Boston, Massachusetts

\*\*Robert Breck Brigham Hospital, Boston Massachusetts

range of disorders represented in table 1-b is extensive, but in no patient was there any reason to suppose that adrenal function was impaired. It may be noted that the response to ACTH does not appear to depend upon the presence of the thymus or the spleen (Patient No 7 and No 14, table 1-b)

The results are displayed graphically in figure 1. There was a mean neutrophil

increase of 104 per cent (range  $-2$  to  $+248$  per cent), a mean lymphocyte decrease of 39 per cent (range  $-4$  to  $-62$  per cent) and a mean eosinophil decrease of 73 per cent (range  $-49$  to  $-99$  per cent). The maximum change in the hematocrit readings (10 patients) was a 2 per cent fall. These findings leave no doubt that ACTH exerts a significant effect upon the quantity of each of these three types of circulating leukocytes. The greater consistency of the eosinophil response is evident in figure 1.

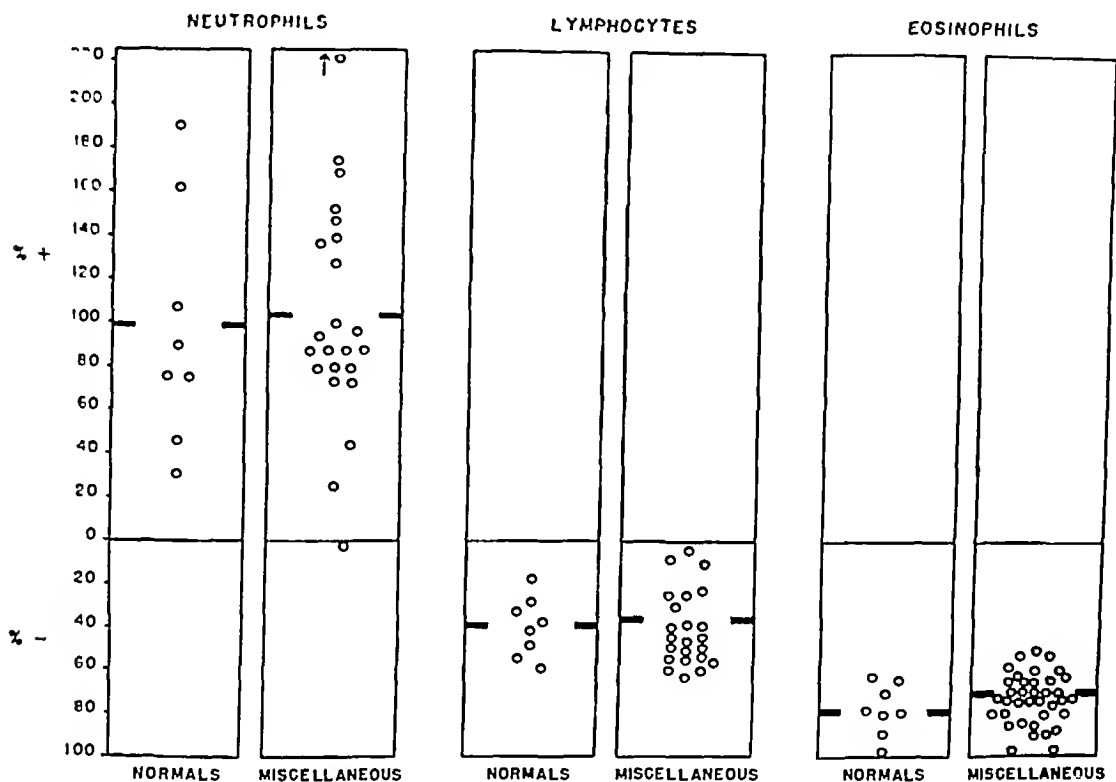


FIG. 1

FIGS. 1, 2 and 3. Each circle represents the change, four hours after injection, in a single subject in the count of each cell type shown, expressed as per cent of the initial count. Heavy broken horizontal bars indicate the average of each group of determinations.

The changes in the leukocyte counts following hormone administration have been uniformly expressed as a percentage of the initial count. This method of expression is particularly desirable in the case of the eosinophils, where the initial counts in otherwise essentially normal individuals may vary widely, and since it can be shown that the cell decrease induced in different individuals by the same dose of ACTH is, in general, proportional to the magnitude of the initial count. This generalization is borne out by table 1 and figure 1, in which it may be seen that all essentially normal subjects exhibit a remarkably consistent eosinophil response to the administration of 25 mg. of ACTH, provided the fall is expressed as a percentage of the initial count. It seems likely that the same generalization applies to alterations in the neutrophil and lymphocyte responses, although here there is much more individual variation, and in any case the greater uniformity of the initial counts make the matter of small importance.

From animal experiments it is established that the ACTH employed has a di-

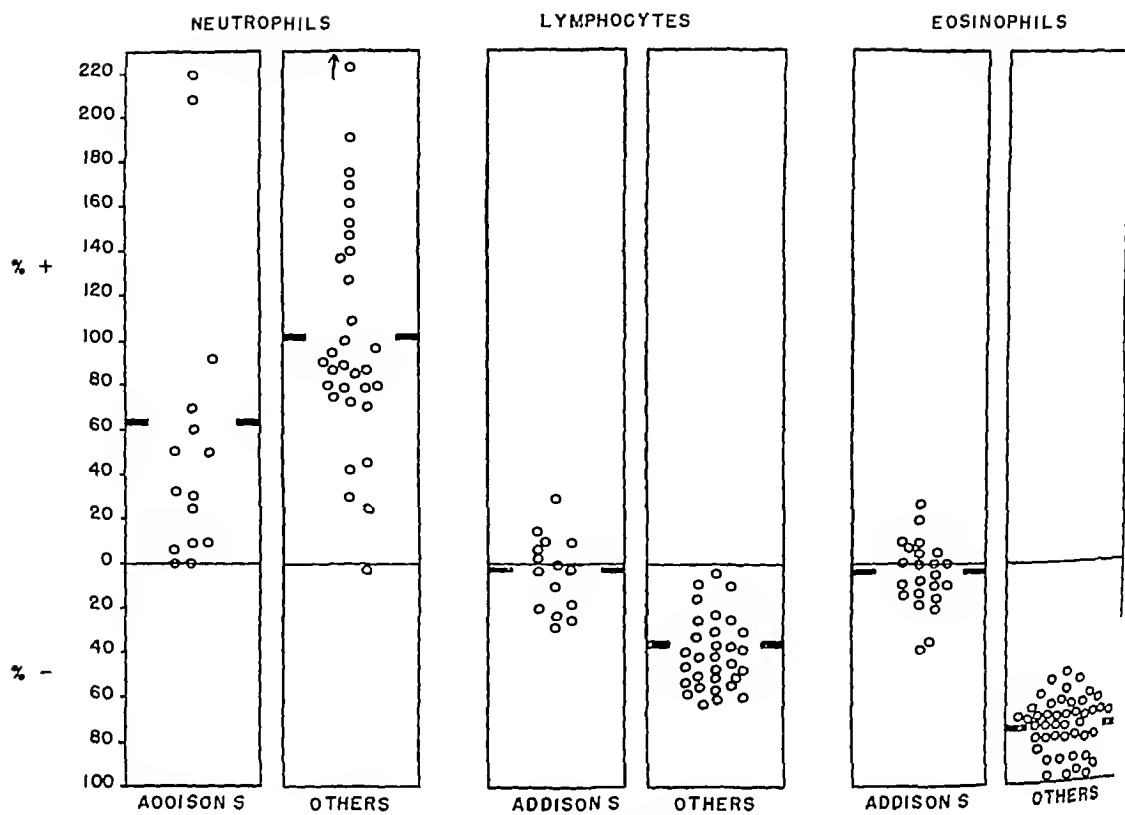


FIG 2 (see legend, fig 1)

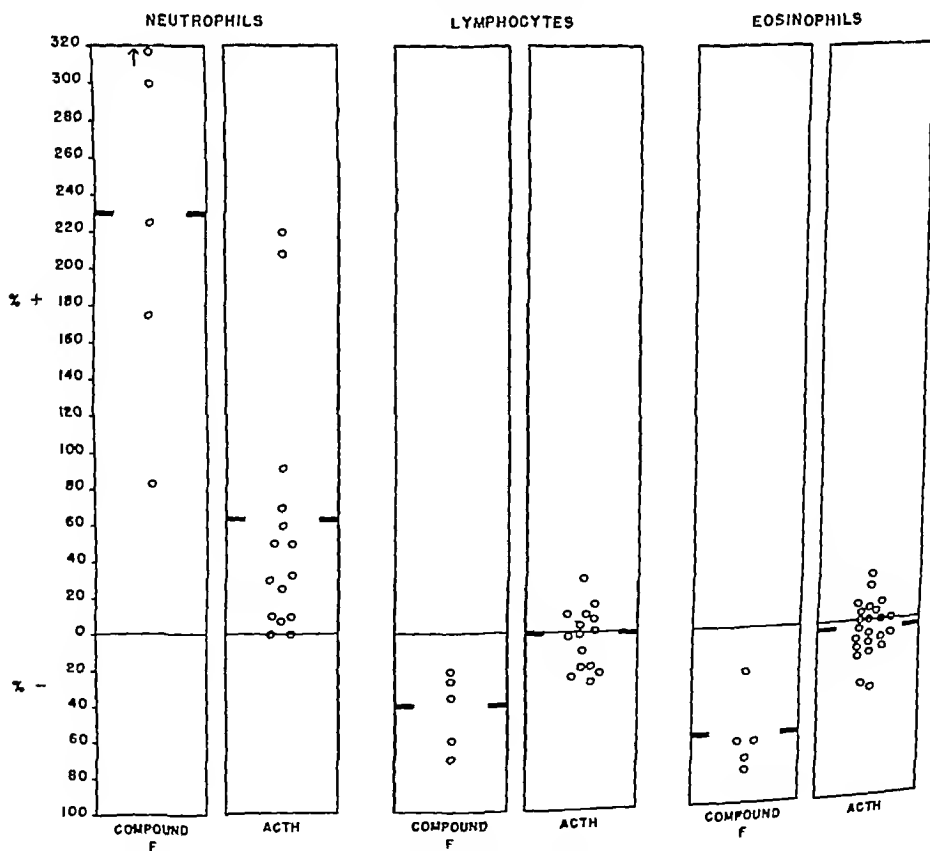


FIG 3 (see legend, fig 1)

rect action in the absence of the pituitary. It is, however, conceivable that some of the results obtained in the study might in part at least be due to pituitary stimula-

TABLE 2 — Effect Upon Leuko cytes of .5 mg. of ACTH in Addison's Disease

Patient	Age	Sex	PBH Hosp. No.	8 AM				12 Noon				% Change		
				WBC	Neut	Lym	Eos	WBC	Neut	Lym	Eos	Neut	Lym	Eos
1	F H	32, M	No 5A144				17				11			-35
2	W C	34, M	No (A41	6650			94	8150			94			0
3	M B	54, M	No (A5	8300			117	10900			147			+26
4	E G	30, M	No 5A808	6200	2540	3225	1257	12000	7800	3720	275	+208	+16	+6
5	F G	51, M	No (A174				140				168			+20
6	J C	52, M	No P1284	5900	2480	3120	113	6250	3180	3160	98	+30	-2	-13
7	E C	13, M	No R5807	10300	5250	4740	137	8800	5200	3430	144	0	-28	+5
8	D S	55, M	No 89024		4480	2750	363		4510	2780	395	0	0	+9
9	F McC	43, M	No 5A487	6100	3110	2135	268	7500	4650	2475	250	+50	+11	-7
10	J P	28, M	No P910	6500	2800	3180	294	8050	5335	2410	269	+92	-24	-8
11	H J	57, M	No 5A457	9450			194	10600			156			-20
12	M E T	22, F	No 5A562	6500	2650	3190	169	8100	4311	3150	169	+60	-1	0
13	M N	46, F	No 5A187	4600	1655	2250	575	5000	1800	2400	575	+9	+7	0
14	R G	33, F	No 5A755	3500	1015	2345	83	4000	1840	2160	80	+71	-8	-4
15	H F	42, F	No P1829	8250	3550	3710	842	8200	4430	2790	770	+25	-25	-9
16	H P	33, F	No 5A840	6100	2680	2930	241	7650	3600	3820	264	+34	+31	+9
17	T H	22, F	No 6A129				114				122			+7
18	H R	35, F	No 5A224				136				116			-15
19	A C	42, F	No 6A302				210				179			-15
20	M K	54, F	No 1A568				265				244			-8
21	M F	49, F	No K6076				306				309			0
22	M B	11, M	No 5A539	6700	1610	4490	363	9000	5100	3530	300	+220	-21	-17
23	N M	51, M	No D1627	7600	3720	3572		8200	4100	3936		+10	+11	
24	C S	44, F	No 5A538	9800	4800	4508		11900	7259	3689		+51	-18	
25	E H	44, F	No 5A206	6600	3630	3100		7450	3870	3200		+7	+3	
26	H M	51, M	No 6A516				117				73			-38
Average												+57	-3	-5

tion by some component of the injected preparation. Since the chief contaminant of the material was posterior pituitary extract, pituitrin in amounts ten times that

present in the usual dose of ACTH was given. This produced symptoms of greater severity than observed with the ACTH preparation, but failed to depress the eosinophil to 50 per cent in three patients who had shown a normal response to ACTH.

TABLE 3 — *Effect of Steroids upon the Leukocytes*

Patient, Age Sex, PBBH Hosp No	Diagnosis	8 A M				12 Noon				% Change		
		WBC	Nt	Lym	Eos	WBC	Nt	Lym	Eos	Neut	Lym	Eos.
F* 20 mg												
J P, 8, M, No P910	Addison s Disease	5605	1510	3190	231	8600	7148	1290	63	+370	-60	-75
V A, 37, F, No 5A495	Addison s Disease	7700	1920	2610	2380	10100	5300	1970	1810	+176	-25	-24
H P, 33, F, No 5A840	Addison s Disease	5750	2070	3450	157	10000	8270	2230	55	+300	-35	-65
E C, 13, M, No R5807	Addison s Disease	12400	5700	6200	570	12550	10400	1883	75	+83	-70	-88
M N, 46, F, No 5A187	Addison s Disease	7500	3648	3420	380	14900	11771	3980	134	+222	-21	-65
Average										+230	-42	-63
DSCG† 30 mg												
J G, 37, F, No 6A630	Addison s Disease	6410	2650	2180	171	6180	2660	2310	150	0	+6	-12
C S, 44, F, No 5A538	Addison s Disease	7400	2910	2180	208	7540	3710	1740	225	+22	-22	+8
I K, 55, M, No B3206	Paralysis agitans				222				198			-11
P O D, 37, M, No 6A285	Chronic nephritis				106				122			+15
A C, 33, M, No 6A397	Psychoneu- rosis				178				274			-18
D P, 36, F, No 6A634	Syphilis				79				81			+3
M C, 37, F, No 6A605	Colitis				192				172			-10
Average												-3.5

\* Crystalline 17-hydroxycorticosterone, kindly supplied by Dr M Kuizenga of the Upjohn Company. Dissolved in warm absolute alcohol, mixed with 1% procaine and injected immediately.

† Desoxycorticosterone glucoside (water soluble) kindly supplied by Dr Ernst Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

### B Effect in Addison's Disease

The leukocyte response four hours after injection of 25 mg of ACTH was also studied in 26 patients with Addison's disease, and the findings are summarized in figure 2 and table 2. There was a mean neutrophil increase of 64 per cent (range

to +220 per cent), a mean lymphocyte decrease of 2 per cent (range +31 to -28 per cent) and a mean eosinophil decrease of 4 per cent (range +20 to -38 per cent). The failure of the blood elements to respond in the usual fashion in this condition is thus very conspicuous, and the conclusion is inescapable that ACTH exerts its action on the leukocytes largely by virtue of its power to stimulate the secretion of the adrenal cortex. It is true that injection of ACTH in patients with Addison's disease, although without significant effect upon lymphocytes or eosinophils, does result in an elevation of the neutrophil count. It is apparent that the injection of this preparation promotes leukocytosis through some action other than

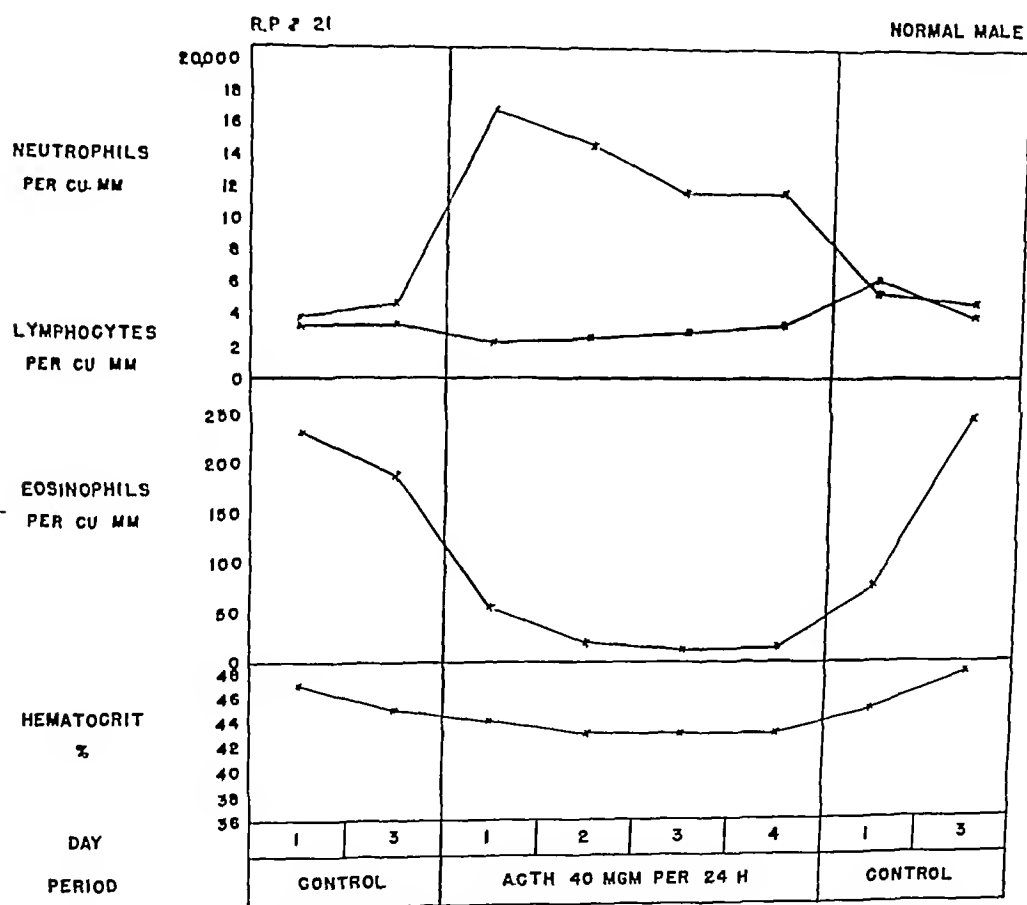


FIG. 4 Effect upon the leukocytes of ACTH, 10 mg intramuscularly every six hours for four days, in a normal human volunteer

stimulation of the adrenal in these patients. It will also be noted, however, that in the presence of a competent adrenal the neutrophil response is of considerably greater magnitude.

It is evident that the distinction between patients with Addison's disease and other subjects is most clear-cut with respect to eosinophils, no patient with Addison's disease showing an eosinophil decrease approaching in magnitude the smallest decrease we have observed in other subjects. This difference between the eosinophil response of the normal and the hypoadrenal subject has been utilized in a diagnostic test for Addison's disease.<sup>6</sup>

## II EFFECT OF A SINGLE INJECTION OF ADRENAL STEROID HORMONES IN ADDISON'S DISEASE

If the failure of these patients to respond to cortical stimulation is due to lack of elaboration of cortical hormones, it should be possible to produce the changes by administration of the proper steroid. Water-soluble desoxycorticosterone was found to be without effect upon the leukocytes in a dosage as high as 30 mg intramuscularly. However, Kendall's Compound F, (17-hydroxycorticosterone),

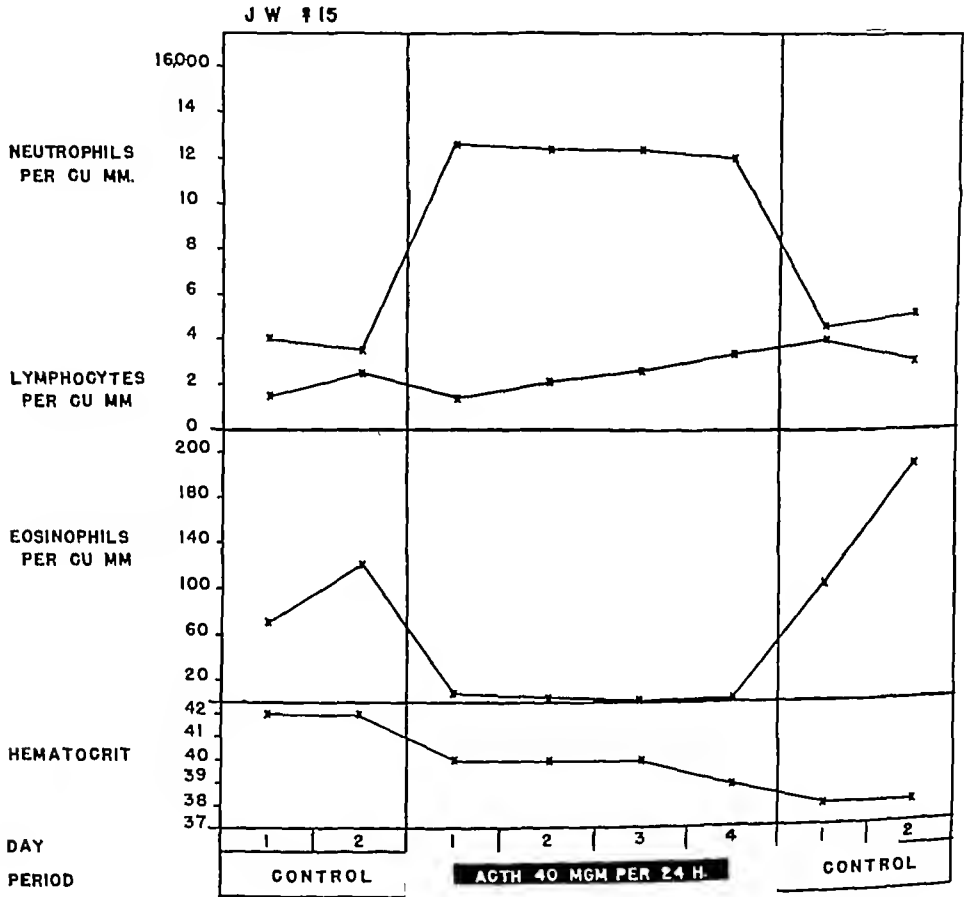


FIG 5 Effect upon the leukocytes of ACTH, 10 mg intramuscularly every six hours for four days,<sup>5,10</sup> in adolescent girl suffering with asthenia

administered to five hypoadrenal patients in a dose of 20 mg intramuscularly, produced the entire pattern of leukocyte changes seen in normal subjects after administration of ACTH (fig 3 and table 3). This finding is in accord with the observations of others<sup>2</sup> in animals that only steroids oxygenated at C-11 appear to be effective in this respect.

## III THE EFFECT OF PROLONGED ADRENAL STIMULATION BY ACTH

The effect upon the leukocytes of more prolonged adrenal stimulation by repeated injections of ACTH (10 mg at six hour intervals), over a period of several days has also been investigated in 2 subjects with normal adrenal function on a constant dietary regimen. The neutrophilia and the reduction of eosinophils are both

striking and well sustained throughout the period of hormone administration, the lymphocytes, however, are not conspicuously depressed after the first day, and in one instance returned within twenty-four hours to their initial level and then increased beyond it. After withdrawal of hormone, eosinophils increase above pre-injection levels, and lymphocytes also reach their highest levels at this time (figs 4 and 5).

In a single subject with Addison's disease, injection of ACTH, 10 mg intramuscularly at six hour intervals for four days, resulted in no significant change in hematocrit or absolute neutrophil or lymphocyte count. The eosinophils exhibited a gradual decrease, reaching a maximum of 25 per cent on the fourth day, and returning promptly to the pre-injection level. In view of the fact that the eosinophil decrease is the most sensitive hematologic index of adrenal secretion, it may be conceived that this small and gradual decrease represented a slight residual capacity of the adrenal to respond to sustained stimulation. This supposition is in accord with the observation that patients with Addison's disease in crisis<sup>16</sup> may show an identical response.

No striking alterations in the morphology of the leukocytes were observed in the stained blood films of the treated individuals.

#### DISCUSSION

Direct evidence has recently been accumulated which indicates that the adrenal cortex influences circulating leukocytes, particularly the lymphocytes. Dougherty and White have reported that adrenal stimulation by adrenocorticotrophic hormone (ACTH) in mice or rats causes a decrease in circulating lymphocytes,<sup>2</sup> tissue lympholysis<sup>7, 8, 9</sup> and an increase in circulating neutrophils,<sup>2</sup> and these findings have been confirmed.<sup>3</sup> The changes in circulating leukocytes were also produced by Kendall's Compound E (17-hydroxydehydrocorticosterone).<sup>2</sup>

Recently, Nordenson<sup>10</sup> reported that the administration of ACTH in a single dose of 150 mg to 2 normal human beings, and a single injection of 150 mg or multiple injections totalling 212.5 mg to 2 patients with lymphatic leukemia, was without significant effect upon circulating blood cells. We are not aware of the appearance of any other publication dealing with the effect of ACTH upon the human leukocyte picture, but Dougherty and White<sup>11</sup> state that the lymphopenic action in the human of ACTH and of certain adrenal cortical preparations has been demonstrated by Darrow and by Hellman.

Physiologic mechanisms brought into play by all types of stress appear to include adrenal cortical stimulation, resulting in excessive secretion of steroids as reflected in increased urinary elimination of 17-ketosteroids and 11-oxysteroids,<sup>12</sup> and by blood changes resembling those obtained by administration of ACTH.<sup>13</sup> Selye<sup>14</sup> has collected an impressive body of evidence pointing to the adrenal cortex as the agency through which many of the known nonspecific reactions to stress are mediated.

That leukocytosis accompanies the widest variety of insults to the organism is a clinical commonplace, it has also been long recognized that in many such conditions there is a reciprocal relationship between lymphocytes and neutrophils.<sup>15</sup> This



nonspecific reaction pattern of the leukocytes is impaired in patients with Addison's disease<sup>16</sup> and in adrenalectomized animals,<sup>17</sup> but in both instances can be produced by the administration of 11-17-oxysteroids. It appears, therefore, to be mediated, to some extent at least, by the adrenal cortex.

As far as we are aware, direct evidence has not previously been put forward that the eosinophils are likewise subject to adrenal control. Nevertheless, a wealth of reports in the literature attests the great variety of clinical emergencies in which a striking diminution of the eosinophils is found. As early as 1893, Zappert<sup>18</sup> reported decrease of these cells as a regular manifestation of a wide variety of infections, and Staubli,<sup>15</sup> in 1910, stressed the reciprocal relation between eosinophils and neutrophils, not only in acute infections but also following injections of nuclein, turpentine, and foreign protein. Starvation,<sup>19</sup> <sup>20</sup>, <sup>21</sup> exposure to cold,<sup>21</sup> operative procedures,<sup>22</sup> peritonitis,<sup>23</sup> and injections of *B. Coli*,<sup>24</sup> sodium bicarbonate,<sup>25</sup> or ammonium chloride<sup>26</sup> have all been shown to cause marked reduction of eosinophils in animals, as have such diverse clinical emergencies in man as intravascular hemolysis,<sup>27</sup> hemorrhage, hypertensive crises, acute congestive failure, ureteral colic and various poisons.<sup>28</sup> Selye<sup>14</sup> has included changes in circulating eosinophils among the manifestations of the "general adaptation syndrome." Evidence presented in this report indicates that the adrenal cortex is important in the production of these changes in man.

The pituitary-adrenal mechanism is only one of a number of factors controlling leukocyte levels. Neutrophilias often seen in certain bacterial infections are of greater magnitude than has been produced by direct stimulation of the adrenal by ACTH and must in all probability require one or more specific neutrophil-promoting factors in addition to the adrenal response to nonspecific stress. Similarly, striking eosinophilias and lymphocytoses cannot be explained as the result of diminished adrenal secretion since the absence of adrenal function in Addison's disease is not as a rule accompanied by alteration in the blood picture of a magnitude comparable, for example, to the lymphocytosis of pertussis or the eosinophilia of certain parasite infestations.<sup>16</sup>

The observations reported in this communication concern only the pituitary-adrenal mechanism which accounts for a part of the leukocytic changes associated with nonspecific stress. Direct information regarding the effect of adrenal stimulation upon the leukocytes in man should, we believe, be of assistance in interpreting the blood changes of certain endocrine disorders<sup>29</sup> and of all clinical conditions which bring nonspecific defensive mechanisms into action.

#### SUMMARY AND CONCLUSIONS

1. Pituitary adrenocorticotrophic hormone (ACTH), when administered in a single intramuscular dose of 25 mg. to human subjects with unimpaired adrenal function, results in a characteristic alteration of the leukocytic pattern. This consists of an increase of circulating neutrophils and a decrease of circulating lymphocytes and eosinophils.

2. The decrease in circulating lymphocytes and eosinophils is contingent upon the stimulation of a functionally competent adrenal cortex, and does not occur in

its absence. The neutrophilic response is present but somewhat diminished in adrenal insufficiency.

3 The entire pattern of leukocytic alterations found in normal subjects after administration of ACTH can be induced in patients with Addison's disease by 17-hydroxycorticosterone (20 mg) but not with desoxycorticosterone glucoside (30 mg).

4 Prolonged adrenal stimulation by ACTH, given over a four day period in a dose of 10 mg every six hours, results in a sustained and striking elevation of neutrophils and depression of eosinophils, the lymphocytes, after an initial depression lasting not more than twenty-four hours, may increase above their initial levels in spite of the continued increased secretion of adrenal hormones.

5 The relation of the adrenal cortex to the characteristic nonspecific leukocyte pattern, observed as a response of the organism to any type of insult, is discussed.

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## THE BLOOD PICTURE IN ADDISON'S DISEASE

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RECENT observations indicate that the adrenal cortex has a controlling influence on certain elements of the peripheral blood. The present study reviews the blood picture in 100 cases of Addison's disease to determine the effect of a deficiency of adrenal cortical hormone upon the blood picture.

### MATERIAL

Hematologic data were obtained from the hospital records of 100 patients in whom the diagnosis of adrenal insufficiency seemed definitely established. In the 22 patients examined at autopsy there was either atrophy or tuberculous involvement of the adrenals. Among the remaining 78 there was increased pigmentation in all but 2. Estimation of the urinary ketosteroids were performed in one-half of these patients and found to be reduced in all cases. In the 63 patients to whom specific therapy was given, the response to treatment was clear cut. Arterial hypotension, heart size, and calcified adrenals by x-ray supplied confirmatory evidence.

### ORGANIZATION OF DATA

Patients were divided into three groups

<i>Group I</i>	<i>Group II</i>	<i>Group III</i>
28 patients	63 patients	9 patients
Average age 43 years	41.5 years	43 years
No specific therapy	Under replacement therapy, either de- soxycorticosterone or adrenal cor- tical extract	Studied before and immedi- ately after treatment

Age distribution for the 100 patients showed 4 in the second decade, 16 in the third, 22 in the sixth and 9 in the seventh. Tuberculosis was considered the causative agent in 42 per cent, whereas 58 per cent were thought to be nontuberculous. In Group I (without treatment) there were 16 tuberculous versus 12 nontuberculous patients, in Group II there was a predominance of 41 nontuberculous to 22 tuberculous patients. The average duration of symptoms of patients in Group I before admission was eight months, subsequent life span was short and frequently complicated by infection. Group II consisted of patients under treatment who had had symptoms over an average of 4.9 years.

### LEUKOCYTE CHANGES IN ADDISON'S DISEASE

Lymphocytosis and neutropenia as well as a general increase in lymphoid tissue have been repeatedly observed in Addison's disease.<sup>1</sup> The recent work of Dougherty and White<sup>2</sup> and others has demonstrated that adrenal steroids control to a considerable extent the circulating lymphocyte level and mass of lymphoid tissue. Reifenstein and Albright<sup>3</sup> have compared the difference in basal blood picture in Addison's disease and Cushing's syndrome and attributed this to the level of adrenal function. In view of these observations and the information obtained by direct stimulation of the adrenal cortex in man, it was of interest to examine more thoroughly the blood of patients with hypoadrenalism to determine what abnormalities might be present not only under basal conditions but under stress conditions as well.

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The most marked alteration in the leucocyte picture was found in those patients under maintenance treatment (Group II), probably attributable to the longer duration of hypoadrenalism. Leukopenia (white cell count below 5,000/cu mm) was observed in 21 per cent of the patients in this group. Lymphocytosis and neutropenia was observed in about one-half of the patients (table 1).

There was no significant difference in the level of circulating eosinophiles by direct count in twenty Addisonian patients from a control group.\* There was, however, an eosinophilia (eosinophiles over 5 per cent) in 14 per cent of the patients in Groups I and II by smear. Although 10 patients had allergic histories, only 2 of these had associated eosinophilia. Allergy usually antedated the Addison's disease and with the exception of one patient with urticaria, all patients showing sensitivity phenomena had adrenal insufficiency of the nontuberculous origin.

It has been shown that adrenal stimulation by pituitary adrenocorticotrophic hormone or a variety of stress conditions produces neutrophilia, lymphopenia and a decrease in eosinophiles in the patient with normal adrenal function.<sup>4</sup> Adrenocorticotrophic hormone does not produce these changes in the Addisonian. The same should be true of stress reactions, either infection or crisis, assuming the hematologic change of stress to be mediated through the adrenal cortex. The blood

TABLE 1 — *Relative Lymphocytosis*

	% over 35%	% over 40% of total WBC
Group I (28 patients)	35	16
Group II (63 patients)	68	48

picture in tuberculous versus nontuberculous Addisonians was identical except for a slight neutrophilia in the former (table 2). The changes in crisis and infection are likewise insignificant except for some decrease in eosinophiles (tables 3 and 4). The absolute lymphocyte count remains remarkably constant and leukocytosis is suppressed. It is evident that the usual leucocyte response to stress is lost in hypoadrenalism.

As an explanation for the drop in eosinophiles, it would seem likely that the stress reaction produces a stronger stimulus for oxysteroid excretion by the adrenal than did the single injection of adrenocorticotrophic hormone. Repeated doses of ACTH have produced in Addison's disease a depression of eosinophiles when single doses have failed. The eosinophile is by far the most sensitive of the blood elements in its response,<sup>4</sup> and since no change occurred in the other elements we might suppose that adrenal excretion was very slight. The adrenocortical extract given in treatment of the crisis, in some instances, may have contributed to the response.

There was no recognizable effect of the maintenance treatment given on the white cell picture. Patients in Group II were carried for the most part on desoxy

\* These data may be found in tables 1 and 2, *Changes in Circulating Leukocytes Induced by Pituitary Adrenocorticotrophic Hormone (ACTH) in Man*, by Hills, A. G., Forsham, P. H., and Finch, C. A. See *Blood*, this issue, pp 756, 761.

corticosterone by injection or by pellet implantation. As previously described, the changes in the blood picture in this group were actually more pronounced than in patients before the advent of specific therapy. Daily maintenance dose of adrenal extract (5 cc Eschatin) and small amounts of Lipo-Adrenal Cortex\* did not change

TABLE 2

	WBC	P	L	M	E	B	Absolute lymph count
<i>Group I</i>							
Tuberculous	8640	60.4	29.4	7.7	2.15	0.25	2540
Nontuberculous	7700	54.9	32.7	7.8	3.9	0.5	2520
<i>Group II</i>							
Tuberculous	6620	52.5	38.5	5.5	3.3	0.4	2550
Nontuberculous	6380	51.3	41.6	3.9	2.9	0.3	2650

TABLE 3 —Effect of Infection on the Blood Picture in Addison's Disease

	WBC	P	L	M	E	B	Absolute lymph count
<i>Group I</i>							
Infection—9 patients							
Temp 100° or over	7455	58	34	7	1		2535
No infection—19 patients	9066	60	26	9	4	1	2357
<i>Groups II and III</i>							
Infection—6 patients							
Temp 100° or over	6191	52	39	7	1	1	2414
No infection—66 patients	6290	51	39	5	4	1	2453

TABLE 4 —Blood Picture in Patients with Crisis  
Average of Ten Cases

	WBC	P	L	M	E	B	Absolute lymph count
Baseline	6140	45	43	6	5	1	2640
Uncontrolled crisis	7835	56	35	6	2.9	0.1	2740
Crisis during treatment	7140	62	30	4.6	3.2	0.2	2140
After crisis, still on treatment	6240	47	43	3.4	6.0	0.6	2680

the blood picture. It has been found<sup>4</sup> that single doses up to 30 mg of desoxycorticosterone have no effect on the blood picture while 20 mg of Compound F (Kendall) results in definite eosinophile and lymphocyte alterations four hours after administration. It seems evident that only appreciable amounts of corticosteroids with the oxygen on C<sub>11</sub> and C<sub>17</sub> have the capacity to produce hematologic changes

\* Whole adrenal cortical extract in oil (Upjohn Company)

## ANEMIA OF ADDISON'S DISEASE

It is frequently difficult to estimate the degree of anemia in Addison's disease because of the variations in plasma volume characteristic of this disease. However, in a group of stabilized patients there was an average hemoglobin of 13.35 grams per cent. In several patients with hemoglobins of 11 to 13 grams, liver and iron therapy did not affect the degree of anemia. On the other hand, an abrupt change in blood volume, as followed treatment in Group III, results in a temporary hemoglobin depression followed by reticulocytosis and return of the hemoglobin to almost precisely the same level as was present before treatment. It would thus appear that with the general metabolic disturbance of Addison's disease, the circulating hemoglobin is set at a lower level.

TABLE 5

	Hgb gm/100 cc	Avg	Range
<i>Group I</i>			
Men	14.2	13.2	9.4 to 18
Women	12.6		
<i>Group II</i>			
Men	13.4	13.3	9.1 to 17
Women	13.3		

TABLE 6

	Hgb/gm	Hct	Rbc
<i>Group III</i>			
Before treatment	13.7	41.6	5.1
After treatment	10.7	32.5	3.8
Averages, % decrease	21.5%	22%	25%

The anemia found in Addison's disease is normocytic and normochromic. In 40 patients of Group II, the average mean corpuscular volume was 92 cubic micra, mean corpuscular hemoglobin 32 micra micrograms, and mean corpuscular hemoglobin concentration was 34.8 per cent. In a small series in which careful cell measurements were made, the same normal cell indices were found. There was no difference in the degree of anemia between tuberculous and nontuberculous patients. In Group I, there was a sex difference not seen in Group II. This might suggest that in the first group, due to the short duration of the illness, the pre-existing sex difference was maintained, and that in Group II this difference had disappeared.

The values for formed elements of the blood in untreated Addison's disease are deceptive because of the markedly contracted blood volume (table 5). Some of the patients, just as do experimental animals in crisis, showed elevated levels of hemoglobin.<sup>5</sup> The actual change in blood volume following treatment may be estimated

from the hematocrit, for during this short time the red cell mass may be regarded as relatively fixed, and fall in hematocrit will be proportional to the increase in plasma volume \* This change averaged over 20 per cent in the 9 patients of Group III, and, as was to be expected, was somewhat greater than that occurring in normal subjects on the same dosage <sup>6</sup>

The anemia may be estimated to be about 20 per cent more severe in the untreated group than in treated patients on the basis of our findings in Group III † This is consistent with the concept that here the bone marrow responds to the relative concentration of hemoglobin in the blood rather than the total cell mass

There was no evidence of increased blood destruction Total bilirubin determinations ranged between 18 and 59 mg per 100 cc in 16 patients with an average of 33 mg per cent In 4 patients, radioactive iron was injected intravenously and the rate measured at which the tagged iron was incorporated into new red cells <sup>7</sup> The iron utilization for hemoglobin production was only slightly less than normal over a period of two to three weeks Serum iron was also within normal limits

### SUMMARY

The blood picture of 100 patients with Addison's disease was analyzed Under basal conditions, there was a tendency toward lymphocytosis and neutropenia Under stress, the blood picture remained fixed The impaired ability of those patients to show leucocytosis and lymphopenia is attributable to their impaired adrenal function While large doses of desoxycorticosterone and maintenance doses of adrenal extract had no effect on the blood picture, 17-oxysteroids produce both neutrophilia and lymphopenia The anemia present in Addison's disease is normocytic and normochromic

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\* There are probably changes in cell volume due to change in osmolar concentration of intra- and extracellular fluid which cannot be evaluated from the present data That these changes are not great is shown by the parallel fall of hematocrit, hemoglobin and red count

† Although average hemoglobin of Group I was 13.2, in Group II 13.3, we can assume a 20 per cent drop following treatment in Group I This has been observed to be followed by a reticulocytosis and return to the initial level



# CARBAMATES IN THE CHEMOTHERAPY OF LEUKEMIA

## I A PROCEDURE FOR SCREENING COMPOUNDS FOR LEUKOTOXIC ACTION

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ALTHOUGH it is fully appreciated that agents possessing a strong leukotoxic action may not in themselves provide an answer to the treatment of leukemia, one cannot fail to be impressed by the uniformity of occurrence of this phenomenon in clinical methods of treatment which have shown some degree of usefulness against this neoplastic disease, i e , x-ray, radium, other radioactive elements, benzene, potassium arsenite, nitrogen mustards, and urethan<sup>1-4</sup> In view of this interesting occurrence, it appeared that information concerning molecules (particularly urethans) which possess cytotoxic specificity, especially upon the hematopoietic system, might in turn aid in the selection of compounds for further study

The present paper presents a simple and rapid technic for rough qualitative determination of the leukopenic action of a given agent, using the mouse as the experimental animal Results of hematologic studies with urethan, nitrogen mustard (HN<sub>2</sub>)\*, x-ray, potassium arsenite, and benzene are reported herein Clinical studies using urethan in the treatment of human leukemia as well as the results of leukopenic assays with some twenty-five structural variations of the urethan molecule are being reported elsewhere

The advantages accompanying the use of a small laboratory animal, such as the mouse, for screening work are obvious, however, lack of well established normals, a small blood volume, possible strain differences, and a considerable variation in the circulating leukocyte count depending on the source of blood (ventricle or peripheral), have been deterrents in the use of mice for hematologic research Attempts have been made in our studies to overcome these difficulties by standardization of the controllable variables

### PROCEDURE

Carworth Farms CFW strain mice, of approximately the same age and weight with equal distribution of sexes, have been used in this study These animals were kept in an air-conditioned animal room and fed a standard diet of Purina dog chow

The first phase of the hematologic screening technic entailed determination of the acute LD<sub>50</sub> of the agent to mice by parenteral administration, using isotonic saline, 10 per cent gum acacia, propylene glycol, or peanut oil as the carrier An arbitrary ten-day observation period was selected and three points on a log-probability plot were considered sufficient for the LD<sub>50</sub> approximation

In the second phase of the study, mice of a given shipment lot were grouped and injected at the LD<sub>50</sub> level and at one-half the LD<sub>50</sub> level Five mice from each group

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\* HN<sub>2</sub> is a designation for the nitrogen mustard, methyl bis (2-chloroethyl) amine

were sacrificed for hematologic study at two and four or four and seven days after injection according to the following plan

Dosage	No. of Animals Injected*	Schedule for Hematologic Examination†	
		4th day	7th day
LD <sub>50</sub>	24	5	5
$\frac{\text{LD}_{50}}{2}$	12	5	5

\* Numbers in excess of requirements for blood studies were injected to make allowances for deaths

† Total erythrocyte count, total leukocyte count, differential leukocyte count, and hemoglobin (Hb) determination were made in all cases. Control studies were made on each lot of mice used

Blood for all counts was obtained by anesthetizing the animal with ether and immediately drawing a fairly large sample (0.3 ml) directly from the heart in the region of the right ventricle. This operation required slightly over one minute. Early in our studies it was learned that etherizing caused a rapid and reproducible increase in the absolute circulating white count of mice.<sup>5</sup> No significant shift in the differential ratio, erythrocyte count, or hemoglobin level was noted. In view of the rapidity of this increase and the lack of any observable shift in the leukocytic formula, it was assumed that the mechanism of this increase was the release of already formed elements from sites of sequestration. Statistical studies on 125 counts of etherized mice (otherwise untreated) showed a degree of consistency compatible with use of this "elevated normal" mean as a base line for leukopenic assays. All results reported herein are on etherized animals.

Blood samples were taken at approximately the same time each day. All mice were sacrificed after exsanguination. Smears and dilutions were made rapidly to prevent clotting.

Summaries of the blood studies of each group of five mice are reported as a mean. Standard deviations ( $\sigma$ ) from the mean have been calculated and are recorded in the case of total leukocyte counts to indicate the degree of internal consistency attained in the various groups.

## RESULTS

Results of blood studies on ten groups of otherwise untreated etherized CFW mice containing five animals each, which provide the base line in this report, are summarized in table 1. Included in this table is a simple statistical analysis of the data obtained.

In order to test the reliability of the screening procedure, groups of mice were given single doses at the LD<sub>50</sub> and one-half the LD<sub>50</sub> level of urethan, nitrogen mustard (HN<sub>2</sub>), x-ray, potassium arsenite, and benzene. The leukopenic action of these compounds and of x-rays is well known, however, it was questionable as to whether single doses would produce a significant response in mice within the chosen observation periods. Toxicity data used in this experiment are listed in table 2.

TABLE 1—Hematologic Studies on Fifty Normal Etherized CFW Mice

	Statistics on Individual Animals				Statistics on Ten Groups of Five Animals Each From Various Shipment Lots			
	Mean	Range	A D	S D	Mean	Range	A D	S D
Total leukocyte count								
Males	7,750	4,000-11,000	1,262	1,730	7,750	5,880-9,050	1,040	1,170
Females	8,150	3,800-13,375	1,907	2,510	8,150	6,130-9,415	1,195	1,473
Males + Females	7,950	3,800-13,375	1,578	2,166	7,950	5,880-9,415	1,119	1,121
Differential count								
Agranulocytes	85.8%	52-97%	7	8.3	85.8%	64-95%	5.5	8.1
Lymphocytes	81.5%	52-94%	—	—	81.5%	61-92%	—	—
Monocytes	4.3%	0-15%	—	—	4.3%	3-7%	—	—
Granulocytes	14.2%	3-48%	7	8.3	14.2%	7-36%	5.5	8.1
Neutrophils	13.6%	3-47%	—	—	13.6%	6-35%	—	—
Eosinophils	0.5%	0-3%	—	—	0.5%	0-1%	—	—
Basophils	0.06%	0-1%	—	—	0.06%	0-0.2%	—	—
Hemoglobin*								
Males	12.5 Gm	7.5†-14.0 Gm	0.8	1.3	12.5 Gm	11.4-13.2 Gm	0.5	0.6
Females	12.5 Gm	11.0-14.0 Gm	0.6	0.7	12.5 Gm	12.2-13.0 Gm	0.3	0.3
Males + Females	12.5 Gm	7.5†-14.0 Gm	0.7	1.1	12.5 Gm	11.4-13.2 Gm	0.4	0.3
Total erythrocyte count (in thousands per cu mm)								
Males	7,284	4,450-8,210	585	795	7,284	6,398-7,894	511	568
Females	7,565	6,000-8,320	563	587	7,565	6,966-7,996	371	358
Males + Females	7,424	4,450-8,320	574	713	7,424	6,398-7,996	391	473

NOTES A D = Average deviation, S D = Standard deviation

\* The Haden-Hausser method was employed for hemoglobin determinations

† Excluding this one low reading, the range was 10.5-14.0 Gm

TABLE 2—Toxicity Data on Known Leukopenic Agents and X-ray

Agent	Vehicle	Route of Administration	LD50	Reference
Urethan	Saline	Intraperitoneal	1800 mg/Kg	This laboratory
Nitrogen mustard (HC <sub>2</sub> )	Saline	Intraperitoneal	3.2 mg/Kg	This laboratory
X-ray			600 roentgens	
Potassium arsenite	Saline	Intraperitoneal	18 mg/Kg	This laboratory
Benzene	Peanut oil	Intraperitoneal	1150 mg/Kg	This laboratory

TABLE 3—Hematologic Studies on Normal\* Mice (CFW Strain) Subjected to Various Treatments

Therapy	No of Animals	Dose	Period After Injection Days	Mean WBC	$\sigma$ WBC	Mean RBC	Mean Hb Gm	Differential	
								Agranulocytes†%	Granulocytes %
Control	50	—	—	7,950	1,222	7,424,000	12.5	86	14
Urethan	5	$\frac{LD_{50}}{2}$	4	3,080	1,229	6,406,000	11.6	88	12
	5	$\frac{LD_{50}}{2}$	7	7,660	4,641	6,702,000	12.9	85	15
	5	LD <sub>50</sub>	4	3,220	2,082	6,572,000	11.5	87	13
	5	LD <sub>50</sub>	7	4,420	2,273	5,976,000	11.7	75	25
Nitrogen mustard (HN <sub>2</sub> )	5	$\frac{LD_{50}}{2}$	4	2,860	432	7,708,000	13.3	74	26
	5	$\frac{LD_{50}}{2}$	7	6,140	2,667	7,474,000	11.6	87	13
	5	LD <sub>50</sub>	4	2,100	1,144	8,242,000	14.1	84	16
	5	LD <sub>50</sub>	7	2,180	1,116	7,238,000	12.4	94	6
X-Ray	5	$\frac{LD_{50}}{2}$	4	3,400	1,343	6,892,000	11.6	97	3
	5	$\frac{LD_{50}}{2}$	7	1,860	755	6,614,000	11.2	94	6
	5	LD <sub>50</sub>	4	1,140	413	6,448,000	12.1	95	5
	5	LD <sub>50</sub>	7	640	287	6,204,000	11.6	98	2
Potassium arsenite	5	$\frac{LD_{50}}{2}$	4	7,660	1,902	6,914,000	11.4	87	13
	5	$\frac{LD_{50}}{2}$	7	7,360	2,190	7,942,000	12.6	93	7
	5	LD <sub>50</sub>	4	3,520	2,080	7,934,000	12.6	96	4
	5	LD <sub>50</sub>	7	4,820	1,251	7,906,000	12.5	92	8
Benzene	5	$\frac{LD_{50}}{2}$	4	6,420	2,264	8,062,000	13.1	95	5
	5	$\frac{LD_{50}}{2}$	7	8,960	4,071	8,106,000	12.8	92	8
	5	LD <sub>50</sub>	4	7,420	1,961	7,686,000	12.0	96	4
	5	LD <sub>50</sub>	7	4,120	1,548	8,006,000	12.6	92	8

\* All animals were etherized before exsanguination, this produced an increase in the formed leukocytes apparently due to their release from sites of sequestration. It is assumed that this rapid release of already formed elements is operative in the treated as well as the control animals.

† In this screening procedure, lymphocytes and monocytes have been grouped under the general heading "Agranulocytes."

Results obtained using the leukopenic therapy referred to above and the four and seven day schedule for hematologic assays are summarized in table 3. Graphic presentation of the leukocyte depression obtained is presented in figures 1 and 2. In

these figures, the normal range refers to the spread observed in grouped means of normal etherized CFW mice

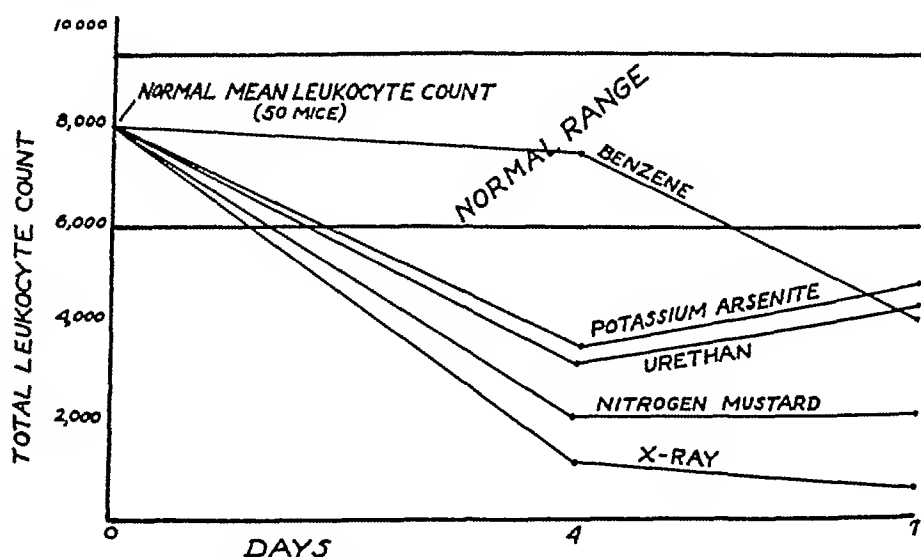


FIG 1 Leukocyte Depression Resulting from Treatment at the LD<sub>50</sub> Level (Each point represents the average of five mice)

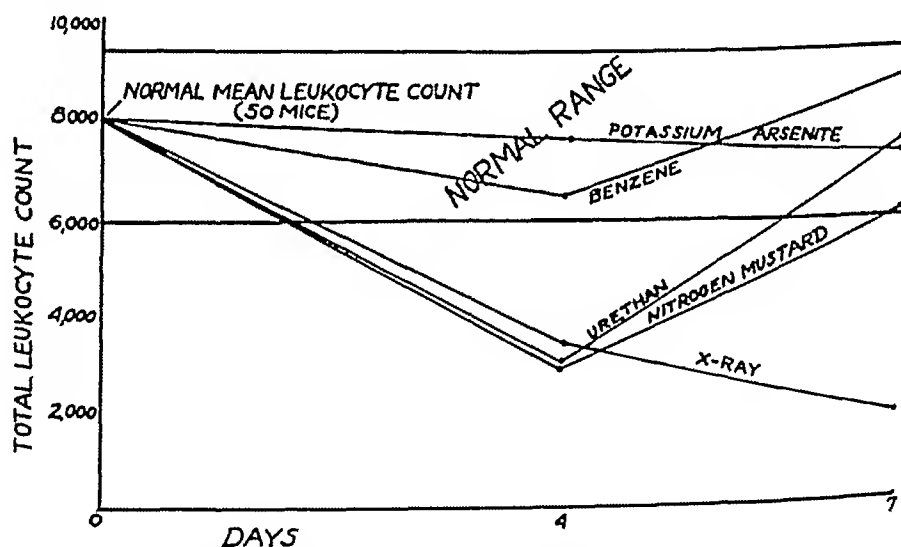


FIG 2 Leukocyte Depression Resulting from Treatment at One-Half the LD<sub>50</sub> Level (Each point represents the average of five mice)

### DISCUSSION

The leukocyte counts of the 50 control etherized mice reported fit well into a normal frequency distribution curve. An additional 75 etherized controls have been studied and the results analyzed and found in good agreement with the present data. In accord with general statistical practice, it is assumed that any group of animals having a mean leukocyte count of plus or minus three standard deviations from the control mean has been significantly affected by the therapy in question. On this basis, there can be little doubt that the leukopenic action of urethan, nitrogen mustard, x-ray, potassium arsenite, and benzene has been demonstrated by the assay.

In this study, comparisons have been made at the LD<sub>50</sub> and one-half the LD<sub>50</sub>

dosage levels, hence the results may be considered to indicate a leukopenic index into which acute toxicity of the agent has been taken into account

Unless a compound shows some specificity toward malignant cell repression or destruction, it may be that leukopenic action should be avoided rather than sought for in chemotherapeutic agents for leukemia. However, until considerable data is accumulated on the life span of leukemic animals treated with leukopenic and non-leukopenic compounds further belaboring this question seems futile. Our laboratory and others are carrying out such leukemic assays.

The somewhat specific mitotic depression observed in urethan leukopenia is paralleled by the cytotoxic action of this compound on epithelial cells of the crypts of Lieberkuehn. This latter action has been reported by Dustin<sup>7</sup> and has been confirmed in this laboratory. It is of particular interest to determine whether the same mechanism is responsible for mitotic depression at both sites or whether certain compounds will demonstrate a specificity correlatable with chemical or physical properties of the drug. Any such specificity should provide interesting data when tested against various strains of leukemia.

The results obtained using this assay on a selected group of urethans, along with comparative results obtained with reference to the mitotic poisoning observed in the intestinal crypts, will be reported in subsequent papers. The same urethans are being assayed for anti-leukemic action against a transplantable myeloid and lymphoid mouse leukemia.

In addition, C<sup>14</sup> tracer studies are being carried out with carbonyl- and ethoxy-tagged urethan in an effort to further elucidate the mechanism of action of this compound.

#### SUMMARY

A simple procedure for screening compounds for leukotoxic action is described. Results obtained on assaying known leukopenic agents such as urethan, nitrogen mustard, x-ray, potassium arsenite, and benzene indicate a reasonable measure of reliability.

#### ACKNOWLEDGMENT

The authors wish to express grateful appreciation to Mr. Ben May of Mobile, Alabama, for support of this project.

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## PARA-AMINOBENZOIC ACID IN LEUKEMIA\*

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M D, AND F H BETHELL, M D

**M**EASURES introduced for the treatment of leukemia have two points of interest. In the first place, any treatment which may mitigate the symptoms and prolong the useful life of persons suffering with a fatal disease of unknown etiology possesses intrinsic merit. In the second, the therapeutic trial of agents which have a demonstrable effect on the growth of leukemic cells is justifiable because of the contributions which may thereby be made to the knowledge of the pathologic physiology of the disease.

In 1944, Flory<sup>1</sup> reviewed the status of those forms of therapy for leukemia which up to that time had been used with varying degrees of benefit. Since then, Paterson and her associates<sup>2</sup> have presented evidence that urethane (ethyl carbamate) is capable of causing improvement, especially in patients with chronic myelogenous leukemia, and Goodman and others<sup>3</sup> have described favorable results obtained with "nitrogen mustard" therapy in some patients with leukemia. The purpose of this communication is to present data which indicate that para-aminobenzoic acid (as sodium para-aminobenzoate) is capable of causing certain definite effects in chronic myelogenous leukemia. Since para-aminobenzoic acid (PABA) is generally considered to be a member of the B-complex group of vitamins<sup>4,5</sup> and is known to be a normal constituent of many food substances,<sup>4</sup> it appears to us that the following observations of its effects in leukemia are of sufficient significance to warrant publication at this time.

Para-aminobenzoic acid has been successfully employed in the treatment of the rickettsial diseases of man<sup>6,7</sup>. It was apparent during the earlier studies of the clinical effects of PABA in louse-borne typhus fever that PABA inhibited the growth of rickettsiae indirectly, that is, by influencing the metabolism of the parasitized cells in some manner.<sup>6</sup> During these studies, it occurred to one of us (C Z) that cells of disordered metabolic function, that is, neoplastic cells, might not be able to adapt to a substrate containing PABA in high concentration. In view of the apparent non-toxicity of large doses of PABA in typhus patients, it was judged safe to test this hypothesis in patients with leukemia.

The present report deals with the results obtained in the study of 10 patients to whom PABA was administered. Five of these subjects had chronic myelogenous leukemia, three had subacute myelogenous leukemia, of whom two were subclassified as erythroleukemia, and two had chronic lymphatic leukemia.

For administration to the patients, para-aminobenzoic acid (PABA)\*\* was placed in solution by conversion to sodium para-amino-benzoate (NaPAB) with sodium

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\*\* The para-aminobenzoic acid used in this study was kindly supplied by Merck and Company, Rahway, New Jersey.

bicarbonate. The final volume was adjusted to make a 10 per cent solution of NaPAB which was administered orally to the patients in 20 cc to 40 cc doses (20 Gm to 40 Gm) every two hours, day and night. The solution was taken most readily in a small amount of fruit juice or ginger-ale. The schedule of therapy employed here is similar to that used in the treatment of certain of the rickettsial diseases<sup>6,7</sup>. The two-hourly schedule is required to maintain a desired blood level since this compound is rapidly excreted in the urine. Purely on an empiric basis, an attempt was made to keep the blood level of NaPAB between 10 and 30 milligrams per cent, although this level was frequently exceeded without any apparent ill effect. In some instances the dosage schedule was modified for patients who continued to take NaPAB at home. They were allowed to double the doses and the intervals between them during the night in order to obtain more rest.

TABLE I

Patient	Diagnosis	Initial white count	Lowest white count	No. days treatment to lowest count	Total Gm NaPAB to lowest count
1 F G	Chronic myelogenous leukemia	427,250	25,250	26	1076
2 K P		230,500	38,000	28	668
3 E N		155,500	24,000	28	1160
4 C W		140,000	20,500	22	998
5 R M		94,500	38,250	15	700
6 L B	Subacute erythroleukemia	23,000	13,000	15	249
7 A C		8,000	2,500	15	580
8 L D	Subacute myelogenous leukemia	41,500	2,200	36	1182
9 C K	Lymphatic leukemia	252,500	101,750	12	566
10 R W		440,000	352,500	18	518

## CASE REPORTS (Table 1)

*Case 1* F G, a 45 year old white salesman, was admitted to the Simpson Memorial Institute on October 31, 1946. He had been well until May, 1946, when he noted loss of weight and progressive ease of fatigue. Symptoms of hypermetabolism were also present.

Physical examination revealed obesity and slight pallor. Several ecchymoses were present over the abdomen and lower extremities. There were no palpable lymph nodes. The liver descended 5 cm. below the right costal margin, and the spleen was greatly enlarged, extending 4 cm. to the right of the umbilicus and down two-thirds of the distance between the umbilicus and symphysis pubis.

On admission the hemoglobin was 11.0 grams per 100 cc., red blood cells 3,600,000 per cubic millimeter, and white blood cells 471,500 per cubic millimeter. The red cell packed volume was 33 per cent and the white cell packed volume was 20.5 per cent. The differential count revealed myeloblasts 0.5 per cent, promyelocytes 1 per cent, myelocytes 21.5 per cent, metamyelocytes 22.5 per cent, band neutrophils 13.5 per cent, segmented neutrophils 35.5 per cent, eosinophils 3 per cent, basophils 1.5 per cent, lymphocytes 0.5 per cent, and hemohistiocytes 0.5 per cent. Bone marrow findings were compatible with the diagnosis of chronic myelogenous leukemia. Urine analysis was negative. Fasting blood sugar was 50 milligrams per 100 cc., the blood cholesterol was 132 milligrams per 100 cc., and the basal metabolic rate was plus 28 per cent.

On November 4, NaPAB was started 2 Gm. every two hours, and increased to 4 Gm. every 150 hours four days later. The patient continued to receive NaPAB for twenty-seven days, taking a total of 125 Gm. During this time his leukocyte count fell from 427,250 to 25,250 per cu. mm. The red cell packed volume rose from 31.0 per cent to 35.5 per cent while that of the white cells decreased from 17.0 per cent



to 15 per cent. The hemoglobin level increased from 10.8 Gm to 11.1 Gm per 100 cc. Throughout the period of medication, there was constant glycosuria of 2 to 3 plus. The blood levels of NaPAB reached 37.2 mg per cent. The patient's spleen decreased in size during the period of therapy, and for some time after its discontinuance. On December 9, when the patient had received no NaPAB for the preceding nine days, his hemoglobin was 12.8 Gm, red cell packed volume 41.0 per cent, white cell layer 2 per cent, leukocyte count 33,050 per cu mm, with differential values essentially as before.

The patient was seen again on December 18, 1946. The spleen had enlarged considerably from the previous examination, and the leukocytes numbered 167,250 per cu mm with 9 per cent myeloblasts.

Three weeks later the leukocytes had increased to 335,000 per cu mm and there was further enlargement of the spleen. Differential count showed 11 per cent blast cells. Platelets were decreased. The hemoglobin was 9.6 Gm. The basal metabolic rate was plus 67 per cent. The patient was admitted for further treatment and was given 4 Gm NaPAB every two hours, day and night. After eighteen days, the leukocytes numbered 141,000, and the hemoglobin was 8.0 Gm. The basal metabolic rate at this time was plus

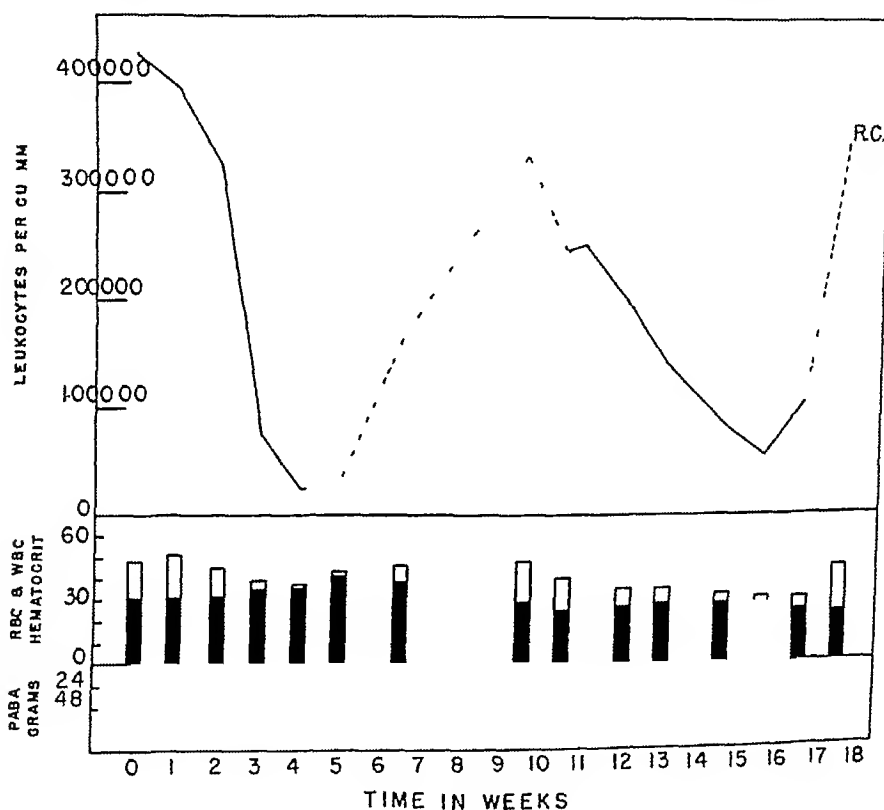


CHART 1. PATIENT F.G. 513293. AGE 45. WHITE MALE. CHRONIC MYELOGENOUS LEUKEMIA

50 per cent, and the patient had gained a few pounds in weight. He was discharged to continue taking NaPAB, 26 Gm daily at home. At the end of two weeks, the leukocyte count fell to 58,500 per cu mm, and the red cell values remained stationary. At this time he had 54.5 per cent blast cells. Thereafter, his leukocytes began to increase despite the continued administration of NaPAB. On February 25, after forty-two days of continuous medication, the white count was 104,750 per cu mm with 59 per cent blast forms. NaPAB was discontinued and the patient felt fairly well for two or three days, but then began to lose ground rapidly. He was readmitted on March 1, 1947. He appeared extremely ill, and was dyspneic, weak, and very apprehensive. The spleen was greatly enlarged, and the leukocytes numbered 344,000 per cu mm with 83 per cent blast forms. The patient became increasingly dyspneic and developed signs of circulatory collapse. Blood transfusion and oxygen were administered, but he died on the following day. Permission for autopsy was not obtained.

This patient received two courses of NaPAB over twenty-seven and forty-two days, respectively. He was given a total of 2,538 Gm of the drug. Glycosuria was noted during both courses of NaPAB administration. Chart 1 illustrates the changes in leukocyte counts and hematocrit values during the period of observation.

*Case 2 K P (Chart 2)* This 69 year old white man first experienced symptoms of exertional dyspnea, weakness, and ease of fatigue in October, 1945. In November, 1945, he was admitted to the University Hospital and the diagnosis of chronic myelogenous leukemia was made. He received a course of total body x-ray irradiation with excellent symptomatic improvement. However, weakness and fatigue reappeared in April, 1946, and he was given more irradiation therapy and several transfusions during May, 1946. He returned on October 1, 1946, and was admitted to the Simpson Memorial Institute. The patient was pale and appeared to have lost much weight. The liver was palpable 3 cm below the right costal margin, and the spleen was greatly enlarged, descending 3 cm below the umbilicus.

On admission the hemoglobin was 7.9 Gm per 100 cc, erythrocytes 2,600,000, and leukocytes 230,500 per cu mm. The red blood cell packed volume was 23 per cent, and the white cell packed volume was 10 per cent. The differential count was as follows: myeloblasts 6 per cent, promyelocytes 3 per cent, myelocytes 16 per cent, metamyelocytes 7 per cent, band neutrophils 26 per cent, segmented forms 22 per cent,

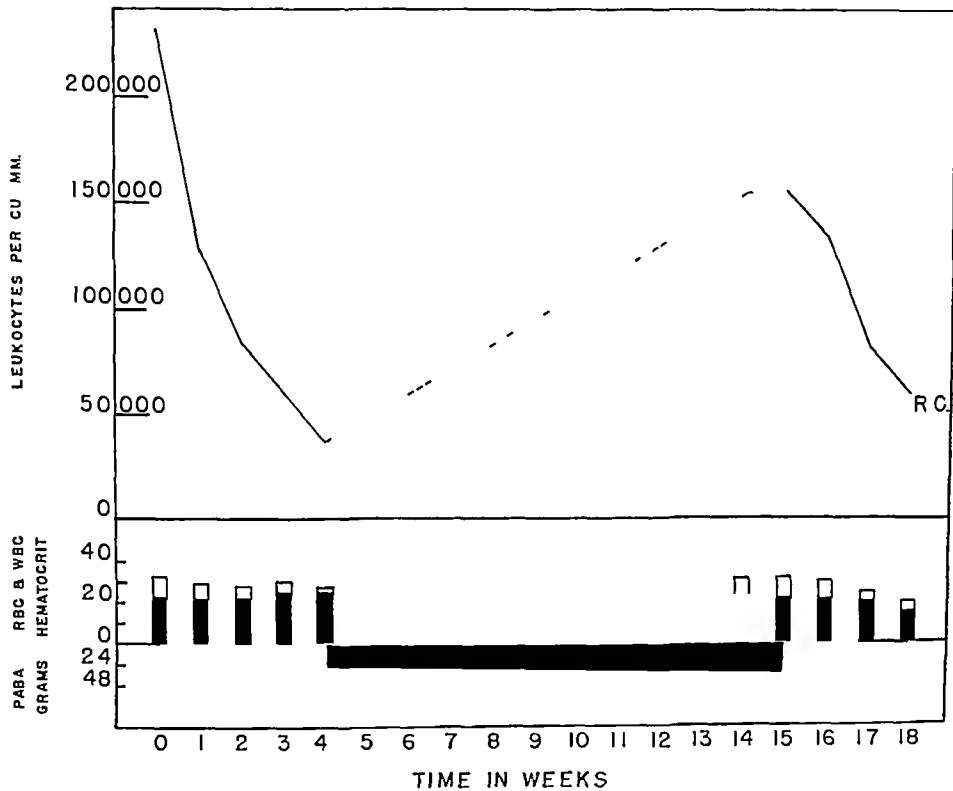


CHART 2. PATIENT K P 580337 AGE 69 WHITE MALE CHRONIC MYELOGENOUS LEUKEMIA

basophils 12 per cent, and eosinophils 8 per cent. The platelets were slightly increased. The fasting blood sugar level was 75 mg per 100 cc. The patient was given 24 Gm of NaPAB daily from October 2 to October 29 when his leukocytes numbered 38,000 per cu mm. He was discharged on October 31 and was not seen again until January 7, 1947.

The spleen now reached a point 6 cm below the umbilicus, and the liver extended 4 cm below the right costal margin. The hemoglobin was 7.5 Gm, the red cells 2,400,000, and the leukocytes 154,950 per cu mm. The red cell packed volume was 23 per cent, and the white cell packed volume was 9 per cent. The differential count revealed myeloblasts 8 per cent, promyelocytes 5.5 per cent, myelocytes 17.5 per cent, metamyelocytes 21.0 per cent, band neutrophils 17.5 per cent, segmented neutrophils 13 per cent, basophils 11 per cent, eosinophils 5.5 per cent, lymphocytes 0.5 per cent, and hemohistiocytes 0.5 per cent. The platelets were increased. Beginning on January 15, the patient received 48 Gm NaPAB daily for three days and 24 Gm daily thereafter. The dosage was decreased because the blood levels attained on 48 Gm a day ranged from 56 mg per cent to more than 70 mg per cent. By February 2, the white cell count had fallen to 60,000 per cu mm and the spleen was slightly smaller and considerably softer than on admission. There was a gain of four pounds. However, the hemoglobin level had fallen to 4.4 Gm. A blood transfusion was started on February 3. After receiving about 100 cc of blood, the patient com-

per cent, segmented neutrophils 26.5 per cent, eosinophils 6 per cent, basophils 4 per cent, and lymphocytes 1.5 per cent. The bone marrow was highly cellular with a great increase in granulocyte forms and reduction of erythropoietic elements. Urine analysis was negative. His fasting blood sugar was 47 mg per cent. Beginning on February 6, the patient received 48 Gm of NaPAB daily for three weeks when the dosage was decreased to 26 Gm per day and the patient was discharged. The leukocyte count decreased from 140,000 per cu mm on February 6 to 20,500 on February 27, and the hemoglobin fell from 10.8 Gm to 8.5 Gm. Fasting blood sugar level was 71 mg per cent on February 25. Glycosuria of 2 to 3 plus was present throughout the period of NaPAB administration. Twelve days later the white count was 22,500 per cu mm, and the hemoglobin had risen to 10.1 Gm. Despite 26 Gm daily of NaPAB, the patient's leukocytes began to increase gradually. On March 21 they numbered 49,750 per cu mm and the amount of NaPAB was raised to 40 Gm daily. A week later the white count was 87,000 per cu mm, but on the following week it had fallen to 47,000 per cu mm. The patient continued to take 40 Gm daily until April 4, when nausea and vomiting forced him to discontinue the medication. When seen again on April 8, the leukocyte count was 46,500 per cu mm, and the urine was free of glucose. By April 23 the white cells had risen to 125,740 per cu mm with a differential count similar to that at the onset of NaPAB therapy. Altogether, this patient received 2,134 Gm of NaPAB over a period of fifty-eight days.

*Case 5.* R. M. was a 71 year old white farmer who was well except for mild symptoms of prostatism until September, 1945, when he became aware of a growing mass in the left upper quadrant of the abdomen. Symptoms of anemia developed and he was admitted to the University Hospital in April, 1946, where the spleen was found to be much enlarged, extending below the level of the umbilicus. The hemoglobin value was 11.6 Gm per 100 cc and leukocytes numbered 75,000 per cu mm. The differential count was typical of chronic myelogenous leukemia. The patient received x-ray therapy to the spleen with subsequent improvement in his general condition and decrease in the size of the spleen. In October, 1946, his spleen appeared to be enlarging and he was bothered by a sense of fullness in the abdomen. On March 27, 1947, he was admitted to the Simpson Memorial Institute. At this time the liver descended 5 cm below the costal margin, and the spleen extended to 6 cm below the level of the umbilicus.

Repeated urine analyses were negative. The urea clearance test showed 71 per cent of normal in the first hour, and 63 per cent in the second hour. Blood urea nitrogen was 18.0 mg per cent and the nonprotein nitrogen was 42.6 mg per cent. The bromsulfalein test for liver function revealed only slight retention of the dye after forty-five minutes. An oral glucose tolerance test was performed after a three day preparatory high carbohydrate diet. The patient received 1.75 Gm of glucose per kilogram of ideal body weight. The results of this test in milligrams of glucose per 100 cc of blood were as follows: Fasting, 68,  $\frac{1}{2}$  hour after glucose administration, 77, 1 hour, 88,  $1\frac{1}{2}$  hours, 117, 2 hours, 103,  $2\frac{1}{2}$  hours, 104, 3 hours, 104, 4 hours, 37, 5 hours, 53. There was 1 plus glycosuria in the second and third hourly specimens, but none at 1 hour, or at the fourth and fifth hours.

NaPAB was administered in dosage of 48 Gm daily, beginning on March 29, 1947. Before treatment the erythrocytes numbered 4,400,000 and leukocytes 94,500 per cu mm. The hemoglobin value was 13.0 Gm per 100 cc. The white blood cell differential count revealed blasts 2.5 per cent, promyelocytes 5.0 per cent, myelocytes 10.0 per cent, metamyelocytes 8.5 per cent, band neutrophils 21.0 per cent, segmented neutrophils 13.0 per cent, eosinophils 20.5 per cent, lymphocytes 1.5 per cent, hemohistiocytes 1.5 per cent, and basophils 6.5 per cent. Glycosuria appeared the day after the NaPAB therapy was started, and persisted throughout the period of treatment.

On April 9, 1947, the oral glucose tolerance test was repeated. Conditions of the test were similar to those before, with the exception that he was now receiving 4 Gm of NaPAB every two hours, and this was continued throughout the test period as well. The fasting blood sugar level was 56 mg per cent,  $\frac{1}{2}$  hour, 78, 1 hour, 111,  $1\frac{1}{2}$  hours, 120, 2 hours, 130,  $2\frac{1}{2}$  hours, 133, 3 hours, 81, 4 hours, 80, 5 hours, 84. There was 2 plus glycosuria before glucose administration, and at the second, third, and fifth hours.

A urea clearance test on April 8, 1947, showed 85 per cent of normal in both the first and second hours. The bromsulfalein test was also repeated at this time, and showed no increased retention of the dye.

By April 12, the patient's leukocyte count had declined to 38,250 per cu mm, and the hemoglobin level had fallen to 9.1 Gm. The differential count was not significantly altered from that at the beginning of therapy. He was discharged on this date to continue NaPAB therapy at home. While in the hospital the patient received 712 Gm of NaPAB over sixteen days. He did not return for his scheduled follow up examination so that additional data are not available.

*Case 6 L B* The patient was a 66 year old housewife who noted weakness, weight loss, and anorexia beginning in March, 1946. The following month she developed furuncle-like skin lesions over face, neck, and chest. For six years she had had episodes of abdominal distress. She was admitted to the Simpson Memorial Institute on August 17, 1946. There was evidence of chronic illness and pallor. Moderately enlarged firm nodes were present in the neck and axillae. The spleen extended 4 cm below the left costal margin and the liver was felt 5 cm below the right lower rib margin. The hemoglobin was 5.6 Gm per 100 cc, and the white blood cells numbered 20,350 per cu mm. Differential count revealed many poorly differentiated cells including 46 per cent blast forms. There were 10 nucleated red blood cells per 100 leukocytes. The red cells were bizarre in form and size. Sternal marrow aspiration showed pronounced abnormalities of both erythropoiesis and granulopoiesis. A diagnosis of subacute myelogenous leukemia was made with the subclassification of erythroleukemia. Folic acid, 10 mg daily, was given for two weeks without any definite effect. On September 4, 1946, the white blood cell count was 23,000 per cu mm, and the hemoglobin level was 6.7 Gm. The differential count revealed myeloblasts 14 per cent, promyelocytes 19 per cent, myelocytes 18 per cent, metamyelocytes 17 per cent, neutrophil band forms 20 per cent, segmented forms 8 per cent, hemohistiocytes 2 per cent, and lymphocytes 2 per cent. On September 5, 1946, NaPAB was started in dosage of 24 Gm daily. Glycosuria was first noted on the day the drug was started and persisted while the medication was continued. Red cell values fell during the period of therapy and two transfusions were given. The drug was discontinued on September 20 after a total of 249 Gm had been taken. Blood values on that day were as follows: erythrocytes 2,300,000 per cu mm, leukocytes 13,000 per cu mm, hemoglobin 5.3 Gm, hematocrit 21.0 per cent, differential count: blasts 14 per cent, promyelocytes 8 per cent, myelocytes 35 per cent, metamyelocytes 20 per cent, band neutrophils 15 per cent, segmented neutrophils 4 per cent, lymphocytes 3 per cent, and hemohistiocytes 1 per cent. By September 25, the white cell count had risen to 43,000 per cu mm. The patient was discharged and died at home a few weeks later.

*Case 7 A C* This patient was a 43 year old Mexican building laborer who noted ease of fatigue in June, 1946. He was able to continue working until October, 1946, when he began to have episodes of vomiting and abdominal pain. About a month later he developed yellowish pallor, progressive symptoms of anemia, and gingival bleeding. He was admitted to the University Hospital on December 6, 1946. The patient was extremely pale, with a sallow skin color but no icterus of the sclerae. There were a few small nodes in the neck and axillae, the liver was felt 3 cm below the costal margin, and the spleen could not be palpated. On December 12, the patient was transferred to the Simpson Memorial Institute where a diagnosis of subacute subleukemic myelogenous leukemia was made. Marrow examination of this patient revealed extensive involvement of the erythropoietic as well as the granulopoietic series, and so led to the secondary diagnosis of erythroleukemia as in the preceding case.

Blood values were as follows: Erythrocytes 2,300,000 per cu mm, leukocytes 8,000 per cu mm, hemoglobin 6.6 Gm per 100 cc, hematocrit 22.0 per cent, mean corpuscular volume 96 cu microns, differential count: myeloblasts 5 per cent, promyelocytes 2 per cent, myelocytes 2 per cent, neutrophils 54 per cent, eosinophils 1 per cent, lymphocytes 35 per cent, hemohistiocytes 1 per cent. There were 20 nucleated erythrocyte elements per each 100 leukocytes counted. Urine analysis was negative. The fasting blood sugar level on December 16 was 71 mg per cent. On that day, NaPAB was begun in a dosage of 48 Gm daily. On the following day glycosuria appeared and continued during the period of NaPAB therapy. The fasting blood sugar level was 61 mg per cent on January 6. The white blood cell count varied considerably, but tended to decrease during the second week of therapy to levels between 2,000 and 3,000 per cu mm which were maintained until the NaPAB was discontinued on January 28. On January 14 the patient developed indurated furuncle-like lesions on the thighs. Three of these enlarged and ulcerated. Secondary infection occurred and the lesions remained stationary as large shallow ulcers. Three 500 cc blood transfusions were given during the NaPAB therapy. Blood values on January 27 were as follows: Erythrocytes 2,000,000, leukocytes 1,800 per cu mm, hemoglobin 5.0 Gm per 100 cc, hematocrit 18.0 per cent, mean corpuscular volume 90 cu microns. The differential count was essentially unchanged from the pretreatment values, except for a relative increase in blast forms.

A series of blood transfusions were given during the following week and the hemoglobin was raised to 12.5 Gm. The white blood cell count increased slightly and was 4,000 per cu mm on February 16 when the patient was discharged. Before discharge, a course of x-ray therapy was given to the ulcers on the lower extremities.

The patient returned on February 26. At this time there had been some improvement in the ulcerating lesions on the legs. The hemoglobin had fallen to 10.3 Gm, while the white cell count had risen to 17,300 per cu mm. There was a striking change in the differential which showed 85 per cent myeloblasts and only 1 per cent mature neutrophils. Late in March the patient was readmitted for a series of plasma transfusions during which the white cells continued to increase in number, reaching 58,500 per cu mm with 71 per cent myeloblasts and 15 per cent mature neutrophils.

A glucose tolerance test was performed on April 9. The fasting blood sugar level was 67 mg per cent. The blood sugar levels of specimens taken at test intervals were as follows:  $\frac{1}{2}$  hour, 130, 1 hour, 236,  $1\frac{1}{2}$  hours, 284, 2 hours, 240,  $2\frac{1}{2}$  hours, 162, 3 hours, 120, 4 hours, 56, and 5 hours, 50. The fasting urine specimen was negative for glucose as were those obtained at 1, 3, 4, and 5 hours. Only the specimen collected at  $2\frac{1}{2}$  hours gave a reaction (3 plus) for glucose. On April 11, NaPAB therapy was reinstituted in a daily dose of 48 Gm for nine days, when it was discontinued because of nausea and cramping abdominal pain. Glycosuria was again noted during the NaPAB treatment, but disappeared four days after cessation of therapy. The white cell count showed no distinct change during the period of therapy, but during the next nine days it rose from 42,250 to 74,000 per cu mm.

*Case 8 L. D.* The patient was a 38 year old white tool grinder who was first seen at the University Hospital in September, 1945, for low back pain of seven years duration. A diagnosis of spondylitis rhizomelique was made and the patient later received a course of x-ray therapy to the spine at another hospital. In October, 1946, he developed symptoms of anemia which became progressively more severe. He was admitted to University Hospital on December 20, 1946. Examination revealed extreme pallor. Lymph nodes were not enlarged and the spleen was not palpable. On the basis of laboratory findings a diagnosis of subacute myelogenous leukemia was made and the patient was given three transfusions, each of 500 cc whole blood. On December 26, the patient was transferred to the Simpson Memorial Institute. At that time blood values were as follows: Red blood cells 2,300,000, white blood cells 41,500 per cu mm, hemoglobin 7.8 Gm per 100 cc, hematocrit 22.0 per cent, differential count: myeloblasts 22 per cent, promyelocytes 2 per cent, myelocytes 18 per cent, metamyelocytes 23 per cent, band neutrophils 4 per cent, segmented neutrophils 5 per cent, eosinophilic myelocytes 9 per cent, eosinophilic metamyelocytes 9 per cent, lymphocytes 7 per cent, and hemohistiocytes 1 per cent. The fasting blood sugar level was 81 mg per cent. Administration of NaPAB was begun on December 28, the patient receiving 48 Gm daily. On the following day glycosuria was noted and this persisted throughout the NaPAB therapy. During the second week of treatment the white count began to fall and by January 11, 1947, had reached 8,300 per cu mm. On January 11, NaPAB was discontinued and on January 14, it was resumed but the dosage was decreased to 24 Gm daily. Six more blood transfusions were given during the period of therapy with NaPAB. On February 1, 1947, the blood values were as follows: Erythrocytes 3,500,000 per cu mm, leukocytes 2,200 per cu mm, hemoglobin 10.2 gm, hematocrit 33.0 per cent, differential count: myeloblasts 23 per cent, promyelocytes 2 per cent, myelocytes 8 per cent, metamyelocytes 8 per cent, band neutrophils 21 per cent, segmented neutrophils 21 per cent, lymphocytes 12 per cent, and hemohistiocytes 5 per cent. At this time the NaPAB was discontinued and the patient was discharged. He had received 1,230 Gm of NaPAB over thirty four days. He died at home about two months later.

*Case 9 C. K.* This patient was a 68 year old white highway construction foreman who had had excellent health until August, 1946, when he developed vague discomfort in the upper abdomen often occurring two or three hours after meals. The following month he lost a considerable amount of weight although his appetite remained normal. In December, symptoms of anemia became prominent and progressed until his admission to the Simpson Memorial Institute on February 3, 1947. Mild symptoms of hypermetabolism had been noted during the three months before admission. Examination revealed extreme pallor, generalized lymphadenopathy, hepatomegaly and splenomegaly. Blood values were as follows: Hemoglobin 4.3 Gm per 100 cc, red blood cells 1,600,000, white blood cells 252,000 per cu mm, red cell packed volume 14.0 per cent, white cell packed volume 5.0 per cent, differential count: atypical lymphoid cells 99 per cent, some with notched nuclei, and polymorphonuclear neutrophils 1 per cent. Sternal marrow aspiration revealed almost complete replacement by the abnormal lymphoid forms. Serum bilirubin was 0.65 mg per cent. On February 6, 1947, NaPAB, 48 Gm daily, was started. On February 17, the white cell count had fallen to 101,750 per cu mm. On February 19, a differential count showed no granulocyte forms except for 1 eosinophil in several hundred cells observed. On February 19, the patient had a sudden onset of chills, fever, and cough and NaPAB was discontinued. He had received 634.0 Gm over fourteen

days. In spite of penicillin therapy and supportive measures, the patient died on February 21. On the day before death, the white count had risen to 180,500 per cu mm. Two plus to 3 plus glycosuria was present during the NaPAB therapy. Autopsy permission was obtained and the pathologic diagnosis was lymphoblastoma of lymphatic leukemia type.

*Case 10.* R. W. This patient was a 77 year old retired white business man who developed an upper respiratory infection late in December, 1946. Early in January he began to notice weakness and malaise and complained of persistence of nasal discharge and a full feeling in his head. A blood count was done and because of the abnormal white count the patient was admitted to the Simpson Memorial Institute on February 25. He appeared much younger than his age, but was definitely pale. There were a few small soft lymph nodes palpable in the neck and axillary regions. The spleen was greatly enlarged, extending 7 cm. below the level of the umbilicus. Blood values were as follows: Red blood cells 2,700,000, white blood cells 410,500 per cu mm, hemoglobin 7.1 gm. per 100 cc, hematocrit 24.0 per cent, differential count: neutrophils 3 per cent, eosinophils 0.5 per cent, monocytes 0.5 per cent, atypical lymphocytes 96 per cent. Sternal marrow aspiration revealed almost complete replacement by the abnormal cells of the type seen in the peripheral blood. The diagnosis was chronic lymphatic leukemia of an atypical type. NaPAB was started on March 1 in dosage of 48 Gm. daily. On March 4, the dose was reduced to 24 Gm. daily. Glycosuria occurred, usually of two plus degree. On March 12, 1947, auricular fibrillation was noted and digitalis was given. On March 13, the patient received a transfusion of 500 cc. of blood. The NaPAB was discontinued on March 19, 1947, because of nausea, temperature elevation to 101 F, and apparent decrease in the granulocytes of the peripheral blood. The blood values on that day were: Leukocytes 352,500 per cu mm, hemoglobin 6.6 Gm., differential count: neutrophils, less than 0.1 per cent, eosinophils 0.5 per cent, and abnormal lymphocytes 99 per cent plus. On March 22 the white blood cells had increased to 528,500 per cu mm. During the following week the patient was given a course of nitrogen mustard therapy (methyl-bis (beta-chloroethyl) amine). On April 7, the white cell count had fallen to 133,000 per cu mm, and there were 4.5 per cent neutrophils.

### DISCUSSION

From the foregoing case reports, it is evident that para-aminobenzoic acid administered in large doses as sodium para-aminobenzoate will bring about a decline in the total leukocyte count of patients with chronic myelogenous leukemia. The fall in white count usually begins during the latter half of the second week of therapy and is most precipitous during the third week. When NaPAB therapy is discontinued, the leukocyte count begins to rise within a few days. Reinstitution of NaPAB administration will again cause a fall in the leukocyte count.

One patient with subacute myelogenous leukemia showed an appreciable fall in the leukocyte count while receiving NaPAB, but little could be evaluated from the results of the administration of NaPAB to the other two patients with subacute myelogenous leukemia of erythroleukemic type.

NaPAB administration was followed by slight decreases in the cell counts of the two patients with lymphatic leukemia, but in these cases therapy was maintained for only fourteen and nineteen days, respectively. It was discontinued primarily because of nausea in one instance, and signs of infection in the other. Both patients then had sharp increases in their leukocyte counts, which were interpreted as evidence that NaPAB had been exerting an effect on the white blood cell level.

Para-aminobenzoic acid in sufficiently high concentration appears to inhibit all phases of granulocyte development in chronic myelogenous leukemia. There was no consistent change in the differential counts of patients receiving this compound and studies of sternal marrow specimens have failed to reveal any blockage phenom-

non'' However, after the first week of treatment, a striking vacuolation of the cytoplasm developed in the myeloblasts and promyelocytes. This change is illustrated in figures 1 and 2 prepared from material obtained by sternal aspiration from patient 3. Figure 1 pictures the marrow prior to the institution of treatment, while figure 2 is of material secured on the thirty-fifth day of NaPAB therapy.

In three of the patients with chronic myelogenous leukemia treated with NaPAB, there was a temporary improvement in the hemoglobin value and the erythrocyte count. There was no apparent effect on the platelets.

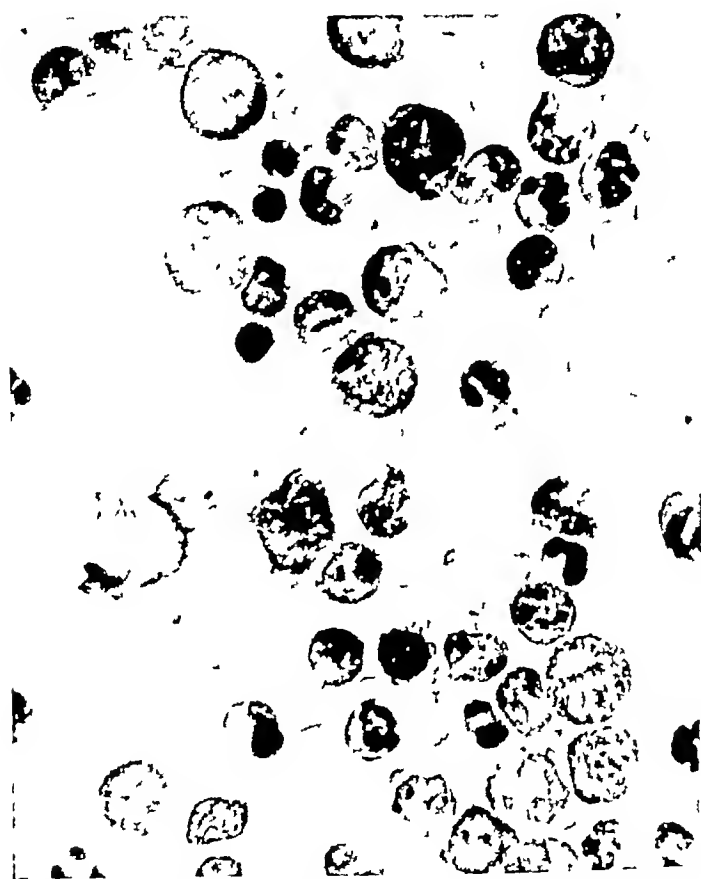


FIG 1 Bone marrow film from patient 3 before treatment showing relative increase in granulocyte forms

Clinically, the patients were not substantially benefitted by the administration of NaPAB. In some instances the spleen decreased in size, the patients gained weight, and there was a diminution of the symptoms of hypermetabolism. However, these effects were not long maintained after the NaPAB was discontinued. In other patients, there was no objective clinical improvement. Three of the patients died in the hospital, and postmortem examination was performed on one (Case 9). The pathologic findings in this case were largely those of the leukemic process. There were no apparent deleterious effects of the NaPAB.

Although the mechanism of action whereby NaPAB in high concentration disturbs leukocyte proliferation and development is not understood, it appears that the factors which cause and maintain elevated leukocyte counts in leukemia are

merely inhibited by NaPAB. The possibility that there is an accumulation of these factors during the period of NaPAB administration is suggested by the striking increase in the number of white cells following the cessation of therapy, and by the more gradual leukocyte increases in patients maintained on reduced dosages of NaPAB (charts 3 and 4).

It is also of interest that glycosuria appeared in all of the patients who received NaPAB. A "reducing substance" was noted in the urines of NaPAB treated patients by the U. S. A. Typhus Commission workers<sup>7</sup> but the nature of this substance was

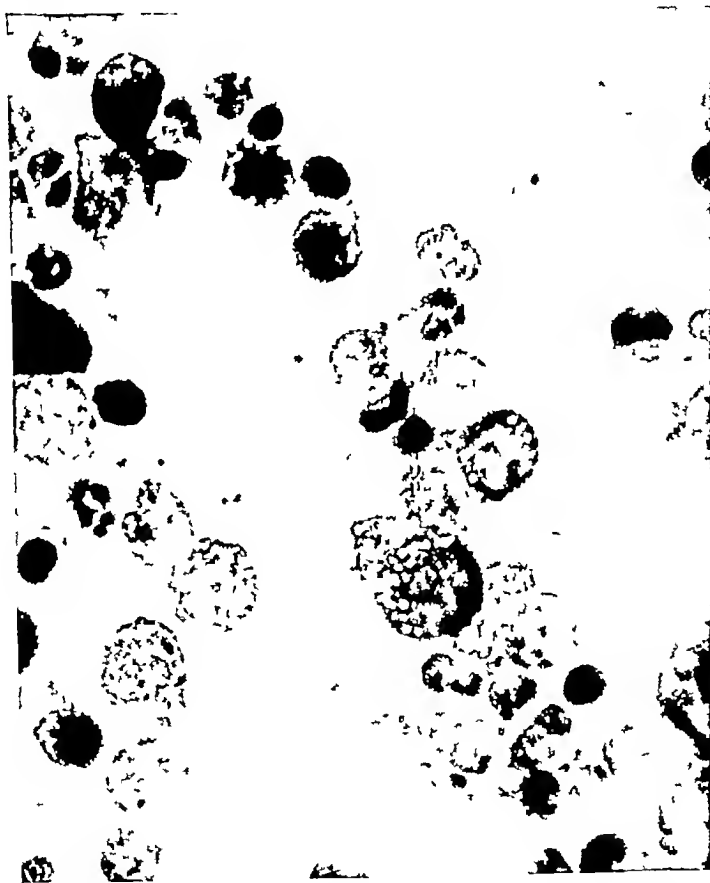


FIG. 2. Bone marrow film from patient 3 after thirty five days of NaPAB therapy. Myeloblasts and promyelocytes show vacuolation of the cytoplasm. The apparent increase in immature cell forms is the result of field selection for demonstration of the vacuoles.

not investigated. Our studies have shown that the reduction of Benedict's solution is caused by glucose, as determined by characteristic osazone reactions. Glucose usually appears in the urine within twenty-four hours after the administration of NaPAB is begun and persists for one to four days after it is discontinued. In a few quantitative determinations, the amounts of glucose excreted ranged from 5.0 Gm to 17.5 Gm per twenty-four hours.

The basis for glycosuria during NaPAB therapy is being further investigated. Fasting blood sugar levels taken before and during therapy have shown no significant differences. Glucose tolerance studies in two of the patients revealed that the pretreatment renal thresholds for glucose were considerably higher than the



level at which excretion occurred while NaPAB was being taken. These observations indicate that the glycosuria is on a renal basis. It does not seem likely that a simple competition phenomenon accounts for the glycosuria and it may be that NaPAB interference with the renal phosphorylation mechanism is the true explanation.

### SUMMARY AND CONCLUSIONS

Para-aminobenzoic acid, administered in large doses as sodium para-aminobenzoate caused a striking lowering of the leukocyte counts in five patients with chronic myelogenous leukemia and in one patient with subacute myelogenous leukemia.

NaPAB caused less definite decreases of the white cell count in two patients with chronic lymphatic leukemia, but the periods of administration may have been too short to obtain maximal effects.

In every instance, there was a prompt rise in the number of leukocytes when the administration of NaPAB was stopped.

Although there was decrease in spleen size in some of the patients, the objective clinical improvement was but slight and temporary.

All patients receiving NaPAB in large doses had concomitant glycosuria, apparently on a renal basis.

It is to be emphasized that NaPAB is not considered a practical adjunct to the therapy of leukemia at this time. Rather, it is hoped that studies of the cellular chemistry involved in the apparent inhibitory action of NaPAB may yield information concerning the disordered metabolism of leukemic cells.

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# ACUTE THROMBOCYTOPENIA INDUCED IN DOGS BY ADMINISTRATION OF URETHANE (ETHYLCARBAMATE)

By W O CRUZ, M D ,\* AND H MOUSSATCHÉ, M D

THE SUBSTANCES inducing thrombocytopenia can be divided into two categories those which act exclusively on the platelets and those which, in addition to this action, act also on the leukocytes and red blood cells

True thrombocytopenia, with no alteration of leukocytes and red blood cells, has been described in man as caused by the administration of organic substances, such as arsenic salts (arsphenamine, neoarsphenamine, etc ), gold salts, and benzene Physical agents, such as x-rays and emanations of radioactive substances, cause thrombocytopenia, always accompanied, however, by leukopenia and anemia

Descriptions have already been made of experimental thrombocytopenia and anemia in dogs, induced by the administration of estradiol benzoate in heavy doses and by injection of antiplatelet serum In previous publications,<sup>1-5</sup> one of us (W O C ) has studied the relation between the decrease of the number of circulating platelets and the occurrence of anemia

This article describes the thrombocytopenic action of ethylic urethane administered to dogs in heavy doses Other blood changes resulting from urethane administration are also described

## METHODS

The volume of the platelets was determined by the Van Allen thrombocytocrit and the results were expressed in ml platelets per 100 ml of blood Details of the hematologic technic used for leukocyte and red blood cell counts are described in a previous paper <sup>6</sup> Urethane was used in a 60 per cent solution in distilled water

## EXPERIMENTAL RESULTS

Preliminary trials with various dosages and routes indicated that the drug exerted some effect on the platelets

Dog 393, 8 Kg

Day of Experiment	Leukocytes 10 <sup>3</sup> /mm <sup>3</sup>	Platelets ml / 100ml blood	Observations
0	10 4		Daily administration of 3 Gm of urethane (0 38 Gm p/Kg ) by mouth
2	17 8	0 58	
5	28 4		
8	16 0	0 40	
12	12 2		Administration of urethane by mouth discontinued
30	2 0		
34			Administration of the same dose of urethane subcutaneously
36	7 8	0 11	Killed by injection of air intravenously Post-mortem examination Hemorrhages in the epiploon and mediastinum No lesions in intestine

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\* Aided by a grant from Dr Guilherme Guinle

*Dog 392, 7.8 Kg*

<i>Day of Experiment</i>	<i>Leukocytes 10<sup>3</sup>/mm<sup>3</sup></i>	<i>Platelets ml / 100ml blood</i>	<i>Observations</i>
0	17.2		Daily subcutaneous injection of 3 Gm of urethane (0.38 Gm p/Kg)
1	31.2		
3	22.0		
8	16.4		Injection area swollen, sensitive to touch, hinder- ing the animal's walk
10	6.4	0	Purpura in areas of injection and other regions of the skin Red blood cells, $4.2 \times 10^6/\text{mm}^3$ Hemoglobin, 10.8 Gm 100 ml of blood Hematocrit, 33%
11	2.2		
12			Found dead in the morning Postmortem exami- nation Medium sized petechiae throughout the intestine, with no preferential zone Pro- fuse hemorrhage in injection site

*Dog 5, 6.2 Kg, Male*

Daily subcutaneous injection (0.1 Gm per Kg) for twenty-two days. On thirteenth day, hemorrhagic zone was noted in inguinal region around one of injection areas, hematologic data showed 6,200 leukocytes per mm<sup>3</sup> and 0.10 ml platelets/100 ml blood. The purpura receded and no hemorrhagic spots were observed.

On the twenty-third day of experiment, urethane was increased to 0.4 Gm per Kg.

On the twenty-eighth day of experiment, cutaneous petechiae were observed.

On the thirtieth day, the animal was killed, after verification of lack of platelets in circulation. Postmortem examination showed presence of many isolated hemorrhagic points throughout the intestine. Hemorrhage in intestinal lumen. Petechiae in both lungs, epiploon and hemorrhage in perirenal tissue.

*Dog 7, 5.4 Kg*

Daily subcutaneous injection of urethane (0.4 Gm p/Kg) for nine days. On ninth day, petechiae on skin of abdomen. On tenth day, hematologic data showed Red blood cells 1,100,000/mm<sup>3</sup>.

Hemoglobin, 2 Gm/100 ml blood. Hematocrit, 8 per cent. White blood cells, 3,000/ml. Platelets, 0. Animal killed. Autopsy showed in the intestine typical picture of hemorrhagic purpura. Pulmonary petechiae.

Three other animals received subcutaneously urethane in doses of 0.4 Gm/Kg body weight for nine days, 0.2 Gm/Kg for eighteen days and 0.4 Gm/Kg for twelve days. They all died and showed little or no purpura. These preliminary observations led us to try experiments which would show more adequately the hematologic changes of thrombocytopenic purpura. Results are given in table 1.

## DISCUSSION

The thrombocytopenic action of urethane has not been previously described. The results referred to above show that this substance has an acute thrombocytopenic action when given to dogs in sufficient quantity subcutaneously. Urethane apparently does not act specifically on platelets, but on all the cell elements of the blood. Leukopenia was also observed in the final phase, and acute anemia without regenerative phenomena, in addition, the platelets disappeared completely from the circulation. The sequence of this picture is similar to that observed when heavy doses of estradiol benzoate are administered to dogs<sup>1</sup> initial leukocytosis, disappearance of platelets, and finally acute anemia. The animal

treated with urethane present a leukopenia which may attain extremely low values in the final phase of the picture

The fall of red blood cells coincides with the appearance of severe intestinal hemorrhages as a consequence of purpuric lesions in the mucosa of the small

TABLE 1.—*Dose of urethane administered 0.4 Gm per Kg*

Dog no	Weight of dog	Day of experiment	Red blood cells $10^6$ ml	Hemoglobin (gm / 100 ml)	Hematocrit %	Reticulocytes %	Leukocytes $\text{mm}^3 \times 10^3$	Platelets ml / 100 ml	Postmortem examination
	Kg								
407-1	9.2	0	5.4	11.6	42	0.5	12.4	0.38	No characteristic signs of purpura
		3	—	12.2	47	0.5	21.5	0.27	
		4†	—	—	—	—	—	—	
408-2	6.7	0	6.2	12.6	48	0.5	16.5	0.39	Few petechiae on intestine. Lung, heart, and skin normal
		4	6.3	12.6	47	0.5	9.2	0.62	
		8*	5.4	10.6	40	0	5.9	0	
		9†	—	—	—	—	—	—	Killed on 11th day of experiment. Petechiae on skin, kidney (cortical zone), lung, perirenal tissue, small intestine. Bloody stools. Hemorrhage at site of injection.
409-3	7.8	0	7.5	15.6	56	0.1	10.4	0.48	
		4	—	13.2	—	—	21.6	0.48	
		8*	6.2	13.4	48	0	5.0	0.12	
		10	5.3	12.2	43	0	2.4	0	
		11†	3.8	6.4	24	0	0.5	0	
410-4	7.2	0	5.9	13.2	50	0.5	15.0	0.45	Petechiae on the lung. Numerous petechiae on the intestine. Large quantity blood in intestinal lumen.
		4	5.1	10.8	38	—	22.0	0.40	
		7	4.8	9.6	29	0	6.8	0	
		8†	—	—	—	—	—	—	
411-6	5.3	0	—	13.8	49	0.5	8.8	0.48	Many petechiae of various sizes on small intestine. Petechiae on lung, heart, and kidney. Copious hemorrhage in area of injection.
		3	6.8	13.4	38	—	19.0	0.38	
		7*	4.8	9.4	34	0	3.2	0.10	
		9	5.4	10.8	37	0	2.6	0	
		10	4.9	8.6	31	0	1.4	0	
		11	3.4	7.6	24	0	1.2	0	
		12	2.5	5.4	17	0	1.0	0	
		13†	—	—	—	—	—	—	

\* Urethane administration stopped

† Coagulation time 7 minutes. Bleeding time Higher than 70 minutes

‡ Died

intestine (figs. 1 and 2). Purpuric lesions similar to those observed in the intestine are frequently found in the lung, cortical zone of kidney, heart, perirenal tissue, epiploon, and skin.

We call attention to the fact that the picture of purpura is established even

when the administration of urethane is discontinued after the fall in the number of platelets. This was the case in dogs 409-3 and 411-6, where the administration of urethane was discontinued on the eighth day of the experiment but the blood changes continued to develop until the final stage, as can be seen in table 1.

The dose of urethane used in the animals listed in table 1 (0.4 Gm per Kg) may approach a dose which would intoxicate the animal prior to the appearance of purpura, this might explain the premature death of two dogs (3 and 407-1).

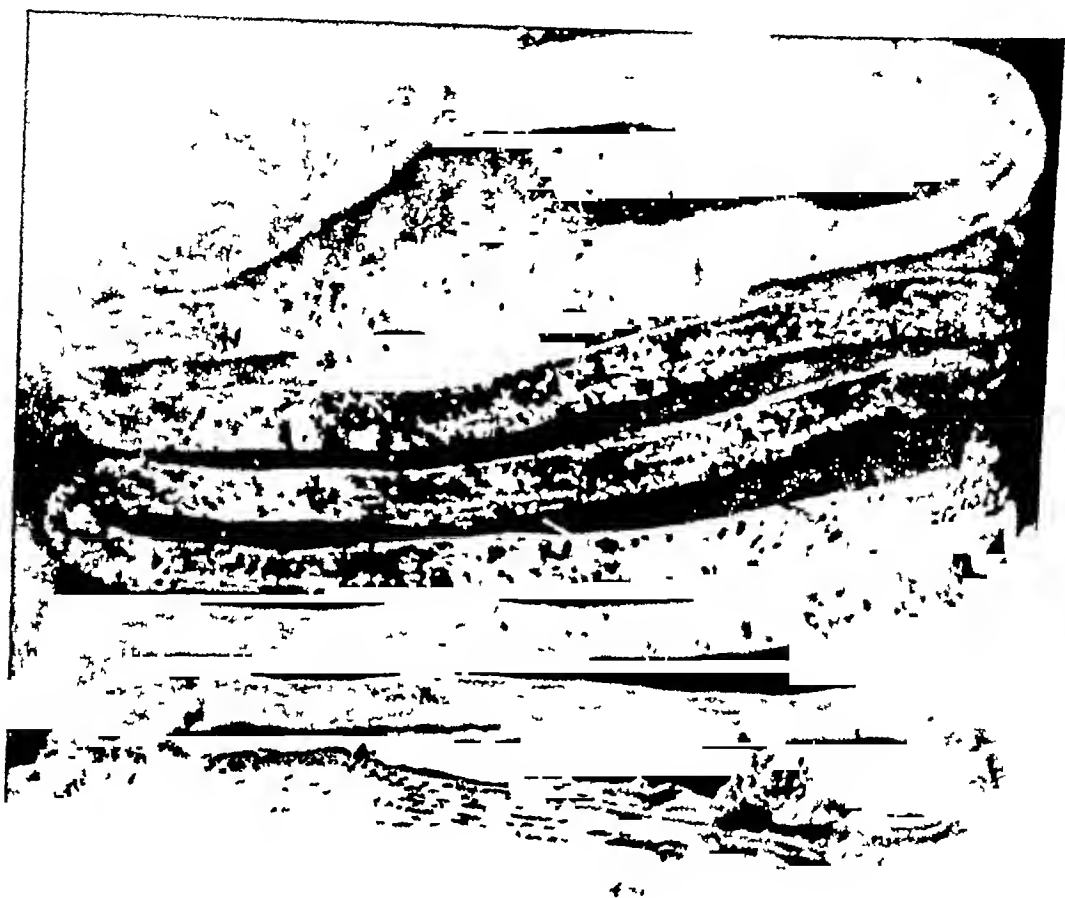


FIG. 1 Dog 409-3

Digestive tract showing extensive purpuric lesions in the small intestine, especially in the jejunum. Stomach, duodenum, and large intestine free of lesions.

The administration of a smaller dose of urethane (0.2 Gm per Kg) for eighteen days did not reveal the typical picture of fulminating purpura. Administration by mouth (0.4 Gm per Kg) for thirty-six days in dog 393 did not produce purpura.

It would still be necessary to discover whether the urethane action is specific for dogs, as is the case with estradiol benzoate. In support of this idea is the fact that urethane was administered to many other species without producing a picture similar to that here reported.

With regard to the mechanism of urethane purpura, it would be well to remember the studies of Krogh<sup>6</sup> and co-workers on the action of urethane on the capillaries. This author, by microscopic observation, verified an intensive dilatation of

capillary vessels of the tongue of frogs submitted to the action of urethane. Also, Landis<sup>8</sup> observed, by introduction of a micropipet in a capillary, an increase of the capillary pressure and permeability in these animals. In the cases of intoxication with high doses of urethane, the capillary pressure may attain values close to arteriolar pressure, the increase in permeability would be connected with the toxic action of the urethane on the capillary wall. It may be supposed that the rupture of the capillary results from damage of the capillary wall combined with



FIG. 2. Dog 411-6

Extensive purpuric lesions in the small intestine, especially in the jejunum

the increase of capillary pressure. Rupture of capillaries was observed by Doljanski and Rosin<sup>9</sup> in studies made on the effects of urethane on the sinusoid capillaries of rat livers.

The known toxicity of urethane for the capillaries and its capacity for inducing purpura could be interpreted as facts in support of the hypothesis of Bedson<sup>10</sup> and others that the primary cause of purpura lies in a change in the capillary endothelium, and not in the thrombocytopenia. However, disappearance of the platelets of the circulating blood some days before the occurrence of purpura in the animals treated with urethane, or, as was shown in previous observations, in animals treated with estradiol benzoate, indicates that the platelets are involved in the mechanism of purpura.

It has not been possible to the present time to determine the primary cause in

the mechanism of urethane purpura, but it seems to us that the whole question of purpura could be attacked with advantage by the use of urethane. Water solubility, low price, as well as easy commercial availability are some of its advantages over estradiol benzoate and antiplatelet serum for the study of experimental purpura.

Because of its leukopenic action, urethane administered by mouth has been used recently<sup>11</sup> in the therapeutic of leukemias. Despite the fact that it is necessary to administer higher doses to dogs than those administered to patients with leukemia, and taking into consideration the fact that subcutaneous injections were used in dogs, it appears to us that in patients treated with urethane it would be advisable to determine periodically the number of circulating platelets.

### SUMMARY

1. Dogs treated for one to two weeks with daily injections of urethane (ethyl carbamate) subcutaneously, in doses of 0.4 Gm per Kg of body weight, presented a typical picture of thrombocytopenic purpura.

2. The pathologic changes consisted in numerous purpuric lesions in the small intestine and a smaller number on the skin, heart, lung, cortical zone of the kidney, epiploon, and, rarely, on the stomach and large intestine.

3. The hematologic changes occurred in the following sequence: leukocytosis, leukopenia and thrombocytopenia, and finally acute anemia coinciding with severe intestinal hemorrhage. In the final phase, the coagulation time was normal and the bleeding time very much increased.

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# A METHOD FOR OBTAINING LIVING LEUKOCYTES FROM HUMAN PERIPHERAL BLOOD BY ACCELERATION OF ERYTHROCYTE SEDIMENTATION

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IN OBTAINING leukocytes from human peripheral blood, use has been made of a physiologic mechanism for accelerating erythrocyte sedimentation. Gray and Mitchell<sup>1</sup> found that the addition of fibrinogen in adequate amounts to heparinized blood causes the red cells to settle so rapidly that they approach cell volume at the end of one hour. We have noted that at one hour, in blood exhibiting an accelerated erythrocyte sedimentation rate, approximately the same concentration of leukocytes remains in the plasma overlying the red cells as is found in capillary blood, and that the differential counts are similar. These leukocytes are alive. Data are presented which show that fibrinogen added to heparinized blood permits the recovery of living leukocytes suspended in their own plasma. This recovery is attended by a minimum of technical difficulty, of physical traumatization, and of erythrocyte admixture.

## METHOD

### *Reagents*

1. Heparin, 100 mg per cent solution, made up in isotonic saline. This solution may be kept in a stoppered bottle at room temperature for several months without significant loss of activity.
2. Fibrinogen solution. This is made up fresh daily by dissolving 310 mg of Fraction I from bovine plasma (Armour) in 10 ml of distilled water. This solution contains approximately 20 mg of fibrinogen per 1.25 ml. Brief incubation at 37 C facilitates solution.

### *Procedure*

- All glassware and needles must be clean and dry.
1. Into a 15 ml tube, pipet 0.4 ml heparin solution.
2. Into a syringe, aspirate rapidly but not forcibly 10 ml of venous blood. Remove the needle and transfer the blood into the tube.
3. Add 1.25 ml fibrinogen solution and mix by inversion.
4. Into another 15 ml tube, pipet 10 ml of this mixture, taking care that the glass above the fluid remains clean, stopper the tube.
5. Slant the tube at a 60 degree angle and incubate at 37 C.
6. After one hour, remove the tube from the incubator and introduce the tip of a 2 ml pipet about 2 cm below the meniscus of the supernatant fluid, taking care not to agitate the erythrocytes.
7. Transfer 2 ml of this fluid to another container. This is a plasma suspension of living leukocytes.

## DATA

Table 1 shows total and differential leukocyte counts and total erythrocyte counts on capillary blood, and on plasma suspension of leukocytes obtained by the method given. Uncorrected erythrocyte sedimentation rates on heparinized venous bloods, determined with Wintrobe tubes at room temperature, are also shown. Data are

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given for consecutive blood examinations of twenty normal individuals (blood donors)

This table shows that after acceleration of the normal erythrocyte sedimentation rate a suspension of leukocytes is obtained in which the concentration and distribution are similar to that found in capillary blood. It also shows that an average clearance of 99.8 per cent of erythrocytes is effected in the suspension. The majority of the residual erythrocytes are present as rouleaux of from five to twenty cells, and

TABLE I — *Total and Differential Cell Counts on Capillary Blood (C) and Plasma Suspension of Leukocytes (S)*

Donor no	Sed Rate Unc mm /hr	RBC		WBC		WBC Differential Count (%)										Ratio of RBC WBC* (Calculated)	
		× 1000 per cu mm				Polys Fil		Polys Non fil		Eos		Lymphs		Monos			
		(C)	(S)	(C)	(S)	(C)	(S)	(C)	(S)	(C)	(S)	(C)	(S)	(C)	(S)	(C)	(S)
1	4	4600	14 5	5 3	4 5	55	61	4	3	2	0	36	36	3	0	868	3 2
2	8	5200	7 8	10 8	10 1	63	63	7	10	3	3	26	24	1	0	481	0 8
3	18	4600	11 8	7 5	6 2	58	60	5	4	0	0	37	36	0	0	613	1 9
4	14	5000	10 0	4 8	4 7	69	73	7	5	0	0	21	22	3	0	1042	2 1
5	4	4900	10 0	6 0	5 4	62	76	10	3	2	0	25	21	1	0	817	1 9
6	20	4300	6 0	6 0	5 4	48	41	6	8	0	0	45	51	1	0	717	1 1
7	13	4500	7 8	5 8	5 2	64	56	3	4	2	3	30	37	1	0	776	1 5
8	17	5200	5 8	5 0	5 0	57	56	1	3	0	0	40	41	2	0	1040	1 2
9	4	4900	9 3	6 5	4 8	60	59	8	5	1	2	28	34	3	0	754	1 9
10	4	4800	10 0	4 6	4 6	63	67	7	0	3	2	25	31	2	0	1043	2 2
11	2	4800	10 0	5 5	5 1	65	66	7	4	0	0	28	30	0	0	873	2 0
12	3	5100	9 7	4 4	5 1	62	60	4	2	0	0	34	38	0	0	1159	1 9
13	3	5100	10 0	5 9	5 6	61	69	3	2	1	1	35	28	0	0	864	1 8
14	4	4900	5 9	10 8	10 9	63	61	4	6	1	1	31	32	1	0	454	0 5
15	2	5200	7 9	6 7	5 1	53	61	5	6	4	3	38	30	0	0	776	1 5
16	2	5300	8 1	6 8	6 7	71	65	3	2	0	0	22	32	4	1	779	1 2
17	17	4700	7 6	10 4	9 8	71	69	3	1	2	1	23	29	1	0	452	0 8
18	3	5500	9 2	6 6	5 9	66	65	1	0	1	1	30	33	2	1	833	1 6
19	12	5000	7 3	8 2	8 1	55	53	2	3	2	2	40	42	1	0	610	0 9
20	10	5300	9 8	6 2	5 9	69	65	1	0	1	1	28	33	1	1	855	1 7
Mean	8	4900	8 9	6 7	6 2	62	62	5	4	—	—	31	33	—	—	790	1 6

\* Average Clearance of Erythrocytes—99.8 per cent

$$100 - \left[ \frac{\text{RBC WBC in (S)}}{\text{RBC WBC in (C)}} \right] (100)$$

only very occasional thrombocytes are found in fresh preparations or stained smears of suspension. Consequently, in many fields observed with high magnification, only leukocytes are seen.

Figures 1 and 2 are photomicrographs, taken two minutes apart, of a drop of suspension obtained by the method given from a normal individual. The changes in

\* This is true because the thrombocytes are for the most part suspended just beneath the cover slip, or are adherent to it, and hence are not in focus while viewing the underlying blood cells. Platelet counts on capillary blood and suspension show a reduction of about 50 per cent in platelet concentration in the suspension.



FIG 1 GRANULAR LEUKOCYTES SUSPENDED IN THEIR PLASMA, RECOVERED FROM NORMAL HUMAN PERIPHERAL BLOOD BY ACCELERATION OF ERYTHROCYTE SEDIMENTATION ( $\times 610$ ) DARK-FIELD ILLUMINATION



FIG 2 SAME FIELD AND MAGNIFICATION AS FIG 1, TWO MINUTES LATER. Note changes in shape and

shape and position of these granular leukocytes, in conjunction with their undiminished refractility as observed with dark-field illumination, afford proof of their vitality

### DISCUSSION

The method given for leukocyte recovery is technically simple. The two reagents used are readily available, no special apparatus is needed, working time is measured in minutes. Minor modifications of the conditions recommended will yield satisfactory results. erythrocyte sedimentation may be carried out at room temperature or with the tube upright, and the sedimenting time is not critical. We have observed, however, that the concentration of fibrinogen salt solution cannot be increased without significant impairment of the vitality of granular leukocytes.

There is minimal traumatization to the leukocytes. The reagents used are substances normally encountered physiologically. Normally circulating leukocytes are obtained, rather than cells accumulating in response to an inflammatory stimulus.

The procedure is applicable to large or small quantities of blood, it has been used successfully with 500 ml. and 1 ml. volumes.

It should be readily adaptable to anaerobic or aseptic technique.

### SUMMARY

A simple method for obtaining living leukocytes from human peripheral blood is presented. It consists of the addition of fibrinogen to heparinized blood. Essentially all the leukocytes remain in plasma suspension and essentially all the erythrocytes are sedimented out at the end of one hour.

### ACKNOWLEDGMENT

The authors wish to express their thanks to Miss Alma Manieri, who did all the blood counts for this study, and to Mr. Antol Herskovitz, who prepared the photomicrographs.

### REFERENCE

- <sup>1</sup> GRAY, S. J. AND MITCHELL, E. B. Effect of purified protein fractions on sedimentation rate of erythrocytes. *Proc. Soc. Exper. Biol. & Med.* 51: 403-04, 1942.

## EDITORIAL

### "SHOT-GUNS"

THE popularity of the numerous and varied shot-gun preparations in the treatment of anemia is one of the features of modern medical practice. Perhaps this points a moral.

Anemia is fundamentally a symptom, being indicative either of a deficiency state, a disturbance of the bone marrow, or of increased blood loss. Anemia per se should not be considered as a disease, and since it is always symptomatic or secondary of something gone wrong in the bodily economy, a thorough-going effort to determine its cause should always be made. The practice of giving mixtures of liver extract, folic acid, iron and vitamin B to patients with anemia in the hope that one or the other of these substances might hit the mark deserves critical comment. Not only is this method slipshod, but it is often wasteful of the patient's finances, and, indeed, in many instances is distinctly dangerous.

Since anemia, if it is actually present (frequently it is not), indicates some sort of abnormal state, it is of prime importance for the physician to attempt to discover the abnormality. This requires a careful history as regards dietary habits, gastrointestinal complaints, bleeding, chemical exposure, hereditary disease and the like. A careful physical examination, followed by an evaluation of the blood picture, will then usually lead to a fairly conclusive idea as to what type of anemia is present, and its possible etiologic mechanisms. A few other studies, including such procedures as stool examinations for occult blood, x-ray examinations, bilirubin estimations, studies of the aspirated bone marrow, etc., will usually lead to a final diagnosis. There can be no doubt that this procedure is time-consuming and requires considerably more trouble than prescribing one or the other shot-gun mixture. On the other hand, a careful study is valuable for the patient, informative for the physician and productive of knowledge which should be helpful in determining whether a simple medication or perhaps a radical surgical procedure is the treatment of choice in a given case.

The prescribing of multi-drug preparations is apt to be wasteful of the patient's finances, for usually some of the numerous medications contained in a given capsule or pill are completely unnecessary. If the patient has an iron deficiency, he needs iron in adequate amounts, not liver extract, not folic acid, not vitamin B complex and not vitamin C. The iron present in the preparation is perhaps adequate, but frequently it is not, and to obtain an optimum amount the patient must often take six to a dozen capsules. There is no good evidence indicating that liver and the various vitamins act as adjuvants to iron. On the other hand, they raise the cost of the preparation five- to twentyfold without otherwise performing a single useful function. Similarly, when a patient has pernicious anemia, what he needs is liver extract or folic acid in optimal amounts and not iron or vitamin B complex. The watchword should be specific medications for specific deficiencies. It may be stated parenthetically that none of the various antianemic preparations *stimulate*

blood formation There are *no* hematopoietic stimulants, only materials which supply a given deficiency, if it is present

Why then may the prescription of multidrug preparations be of distinct danger? Suppose, for example, that the patient has hypochromic anemia due to unrecognized chronic bleeding from the bowel, administration of a shot-gun preparation may well relieve the anemia, at least to some extent and for a little time, but meanwhile the neoplasm or other condition causing the bleeding may continue relentlessly and by the time it becomes obvious, radical surgery may be of no value Or suppose the patient has pernicious anemia and is given six pills or capsules daily containing a small amount of liver extract This amount may well initiate a minor therapeutic response, largely hematologic, but will hardly be sufficient to protect the patient against neurologic complications

Too many attractive preparations for the treatment of anemia are on the market Their worth to the practising physician and to the patient is often highly questionable There is need for cooperative action on all fronts so that the patient may be protected from useless, expensive, and potentially harmful medications This will require not only constant vigilance on the part of the physician but a highly developed ethical sense on the part of the pharmaceutical manufacturer as well as a firm determination on the part of the publisher of medical periodicals to accept only those advertisements which do not overstate the case for a given preparation Where to draw the line is sometimes difficult, but if any one of the groups concerned falters in its determination to give the patient the best possible chance, the shot-gun habit will continue, and will sooner or later back-fire Although a certain degree of financial sacrifice is bound to occur through well-controlled advertising selection, there can be no question that a frontal attack on this problem by practising physicians, pharmaceutical manufacturers and medical publishers would surely redound to the credit of all concerned and prove to be of great help to the ultimate consumer, the patient Through the cooperation of the publisher and the members of the editorial board, this Journal has been making efforts, oftentimes with great difficulty, to screen advertising copy very carefully It will continue to do so

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# ABSTRACTS

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## RADIATION AND RADIOACTIVE ISOTOPES

MEDICAL APPLICATIONS OF ARTIFICIAL RADIOACTIVE ISOTOPES *K. A. Evelyn* From the Department of Medicine, McGill University, Montreal, Quebec, and the Hypertension Research Committee, Massachusetts General Hospital, Boston, Mass. *Canad. M. A. J.* 56: 547-554, 1947

The author presents a remarkably lucid review of some of the fundamental principles pertaining to the medical use of artificial radioactive isotopes. Definitions of the terms isotope, radioactivity, hard ray, soft ray and rate of disintegration will be particularly helpful to the uninitiated. The uses of the Lauritsen electroscope, Geiger counter and the radio-autograph technic are briefly explained. The present status of tracer research is summarized with especial reference to some of the technical difficulties involved. Characteristics of some of the more important artificial radio-elements are given in easily understood tabular form. Data are included on radio-hydrogen, -carbon, -oxygen and -nitrogen because of their potential value, despite the fact that various difficulties are delaying their widespread use.

Discussion of the use of artificial radio-elements for internal radiation therapy is largely confined to radio-phosphorus and radio-iodine. The health hazards involved in the use of artificial radioactive isotopes are properly emphasized.

L E Y

THE USE OF ISOTOPES IN MEDICAL RESEARCH *J. H. Lawrence* From the Donner Laboratory of Medical Physics, University of California, Berkeley, Calif. *J. A. M. A.* 134: 219-225, 1947

This report is a brief, lucid presentation of certain of the fundamental principles in the applications of isotopes in medical investigation and therapy. Isotopes are forms of a given element which have different atomic weights, certain isotopes not only have different weights, but also emit rays of various types, and are, therefore, known as radioactive isotopes. The amounts of such substances can be determined by means of the Geiger-Muller counter and similar devices. Artificially radioactive elements can be produced either by use of the cyclotron (which bombards given elements with positively charged nuclear particles) or by means of the uranium pile (which bombards elements with slow, uncharged neutrons). In general, such isotopes have two uses for medicine: (1) for therapeutic irradiation, and (2) for tracer studies of the metabolism of the particular element.

Lawrence tabulates a number of radioactive substances in use in research, and discusses three principal technics used in such studies: (1) In the *autoradiographic technic*, a tracer dose of a substance is given, tissues are sectioned at various intervals and placed against photographic film, these photographs are then examined to determine which organs have taken up the substance. (2) In the *in vitro technic*, a radioactive substance is given, and samples of blood, urine, etc., are taken at various intervals and measured for radioactivity. (3) In the *in vivo technic*, a Geiger counter is placed successively over various parts of the body after administration of a radioactive substance, and the uptake by various organs thus determined. Cobalt, for example, is taken up largely by the liver, and iodine by the thyroid gland.

This article is an excellent brief introduction to the methods and principles of radioactive research in medicine.

S E

THE HEMATOLOGICAL EFFECTS OF IONIZING RADIATIONS IN THE TOLERANCE RANGE *L. O. Jacobson and E. K. Marks* From the Metallurgical Laboratory, University of Chicago, Chicago, Illinois. *Radiology* 49: 286-298, 1947

The plutonium project was that section of the Manhattan District which had the specific task of preparing and purifying plutonium for use in atomic bombs, according to the general formula,  $U^{235} + \text{neutron} = \text{chain reaction}$ , which gives a neutron which bombards  $U^{238}$  (in the pile) to give plutonium. Studies of the physical, biologic, and clinical effects of irradiation were made throughout the work. It was the task of Jacobson and Marks to study the hematologic data in individuals exposed to irradiation within the 'permissible' or 'tolerance' range, i.e., up to 0.1 roentgens per day. Some of their results are summarized in this report.

Rabbits, mice, and guinea pigs were exposed to irradiations under conditions meant to duplicate the laboratory exposure which scientists and their assistants received in radiation laboratories. The animals were given whole body chronic exposure to external radiations, in various groups (0.1, 1, 2, 4, and 8.8 roentgens for eight to twenty-four hours daily, six days a week, for over three years). Tolerance doses (0.1 r/day) had no effect on the blood. Doses of 2.2 r/day or more resulted in changes in all 3 species, including lymphocytopenia (the earliest change), anemia, and, in the highest doses, death. No lymphocytosis, eosinophilia, or monocytosis occurred in any species at any dosage levels. It was of interest that, in female mice, ovarian tumors developed in doses of even 0.1 to 1.14/day, i.e., *in the absence of hematologic changes*.

The hematopoietic system of man was found to have the same sensitivity to irradiation as that of the guinea pig and the dog. When exposed to chronic tolerance doses, man sometimes developed leukopenia due to lymphocytopenia, and macrocytic anemia. These changes always disappeared, but in varying intervals of time. All radioisotopes studied produced lymphocytopenia as the first indicator of effect, even in the absence of histologic evidence of changes in the lymphatic tissue of the body (animals). However, as demonstrated in the case of mouse ovarian tumors, pathologic changes might occur in the absence of blood and hematopoietic tissue changes at autopsy.

In studies on laboratory personnel, rarely could peripheral blood changes, notably leukopenia, be attributed to radiation. Both exposed and nonexposed groups often developed similar blood changes from extraneous causes (e.g., rubella). In other words, no blood changes could be found which occurred in the tolerance range of radiation exposure, and it was finally concluded that the appearance of hematologic changes should be considered very serious and indicative, probably, of overexposure beyond tolerance doses.

S.E.

THE TREATMENT OF POLYCYTHEMIA VERA BY SPRAY IRRADIATION. W. Richardson and L. L. Robbins. New England J. Med. 238: 78-82, 1948.

This report summarizes sixteen years of experience with x-ray therapy in polycythemia vera. Spray irradiation is given with a target distance of 215 to 250 cm, over a field from neck to knees, a kilovoltage of 200, 0.5 mm copper and 1.0 mm aluminum filtration. Daily dosage varies from 20 to 30 r measured in air and the total dose is approximately 300 to 500 r in any one series of treatments divided between anterior and posterior fields. Treatment is stopped if white blood count falls below 5-6000.

Twenty-eight cases of polycythemia vera treated in this manner are reported with a five and one half year average follow-up. Radiation sickness is minimized in this report. Results obtained compare very favorably with the best results achieved by radioactive phosphorus with remissions of as long as eight years following one course of treatment. One patient developed aplastic anemia. No patients developed leukemia or a leukemoid picture. This latter is of particular interest in view of recent case reports of acute leukemia following radioactive phosphorus treatment.

C.A.F.

PRIMARY AND SECONDARY VACUOLES IN THYMIC CELLS EXPOSED IN VITRO TO X-RAYS. R. Schrek. From Tumor Research Unit, Veterans Administration Hospital, Hines, Illinois. J. Cell and Comp. Physiol. 22: 203-224, 1947.

When suspensions of finely minced rat or rabbit thymus are incubated at 37°C, they undergo certain aging and degenerative processes which are manifested as vacuolar-like structures by dark-field illumination. In similar suspensions irradiated with 1000 r, the development of these structures is accelerated. Irradiation also decreases the number of viable cells as indicated by their resistance to a 1:2000 solution of eosin in Tyrode. Two types of vacuoles were observed in dark-field preparations. The primary ones were either single or multiple, small or large, usually round and clearly outlined. Secondary vacuoles were

large, dark, and round or oval Feulgen preparations revealed that the primary vacuoles were intranuclear and that the secondary vacuoles actually represented pyknotic nuclei. When the latter were extruded they behaved as spherical fluid globules immiscible with water. A hypothesis of the action of x-rays on lymphocytes is discussed.

O P J

## HEMORRHAGIC DISEASES AND BLOOD COAGULATION

**HEPARINEMIA (?) AN ANTICOAGULANT IN THE BLOOD OF DOGS WITH HEMORRHAGIC TENDENCY AFTER TOTAL BODY EXPOSURE TO ROENTGEN RAYS** *J G Allen, M Sanderson, M Milham, A Kirschon, and L O Jacobson* From the Department of Surgery, University of Chicago, and the Argonne National Laboratory, Chicago, Ill. *J Exper Med* 87 71-80, 1948

Dogs exposed to 450 units of roentgen irradiation over the whole body developed infection and hemorrhage associated with neutropenia, thrombocytopenia and prolonged clotting and bleeding times. The prothrombin time remained normal until about twenty-four hours before death, and the concentrations of calcium, phosphorus and magnesium in the blood were not altered. Actual measurements of fibrinogen were not made, but the formation of clots was interpreted as evidence of the presence of fibrinogen. Delay or absence of clot retraction was related to platelet deficiency. The blood of irradiated animals appeared to gel before an actual clot was formed, and the gelled blood could be reverted to a fluid state if shaken before a solid clot formed.

Toluidine blue and protamine sulfate, substances known to inhibit the action of heparin, restored clotting time to normal and were the only agents effective in arresting hemorrhage in the dogs. Transfusions, vitamin K and vitamin C did not alter the hemorrhagic state. It is concluded that the bleeding tendency of the irradiated dogs was due, at least in large part, to the presence in the circulation of an anticoagulant whose properties were indistinguishable from those of heparin. The material isolated with difficulty from the blood of the dogs appeared in no way dissimilar from a standard sodium heparin salt, but was classed as heparin-like because the exact identity of heparin itself is unknown.

The contention that bleeding was not due to thrombocytopenia alone was supported by the observations (1) that the reduction of platelets did not always coincide with the onset of bleeding, and (2) that toluidine blue and protamine sulfate stopped the tendency to bleed but did not elevate the platelet count.

In connection with this report, the highly beneficial effect of rutin in irradiated dogs described by Rekers and Field (*Science* 107 16-17, 1948) is of considerable interest. It appears that loss of vascular integrity may be another factor to be considered in exploring the mechanisms responsible for hemorrhage in irradiated animals.

L E Y

**HYPERHEPARINEMIA THE CAUSE OF THE HEMORRHAGIC DISEASE PRODUCED BY TOTAL BODY EXPOSURE TO IONIZING IRRADIATION** *J G Allen* From the Department of Surgery, University of Chicago. *Fed Proc* 6 68, 1947

During the course of investigations on the effects of atomic radiations on living tissues, it was found that such ionizing radiations often resulted in multiple petechiae and death of a hemorrhagic diathesis. The reduction in platelets was not the entire answer to the development of the hemorrhagic tendency, for these patients showed, in addition to a reduction in platelets and an increase in bleeding time, a marked prolongation of the coagulation time. Further studies disclosed that the blood often contained an anticoagulant, the nature of which seemed to be heparin, for anti-heparin materials (toluidine blue, protamine) restored the coagulation time to normal both in vivo and in vitro. The return of the coagulation time to normal occurred independently of the platelet level, which usually remained low. Calcium, phosphorus, magnesium, prothrombin time, and fibrin formation were all normal in these cases. Finally, when dogs were exposed to ionizing radiation over the body, their plasma was found to contain an anticoagulant having the activity of heparin.

The mode of production of excessive plasma heparin by body irradiation remains to be determined. In the light of such studies, the reported effect of x-radiation in reducing the coagulation time of the blood of patients with hemophilia (Ostro and Macht, *South M J* 39 860-867, 1946) must be held in abeyance until confirmatory evidence is presented. Certainly, it would be strange if the hemophilic



patient, who already, according to one school of thought, harbors an excess of heparin in his plasma should behave in an opposite manner to the normal individual in the response to irradiation

S.E.

**CLOTTING DEFECT IN HEMOPHILIA DEFICIENCY IN A PLASMA FACTOR REQUIRED FOR THROMBOPLASTIN LIBERATION FROM PLATELETS** *K M Brinkhaus* From the Departments of Pathology, State University of Iowa and University of North Carolina *Fed Proc 6 389-90, 1947*

The author suggests that a plasma factor is involved in the release of thromboplastin from platelet and that the factor is deficient in patients with hemophilia. Normal citrated plasmas which coagulate slowly on recalcification were prepared by prolonged centrifugation at 2°C in glassware treated with silicone. Such plasmas were much less effective than ordinary plasmas in correcting the delayed clotting of platelet-free hemophilic plasma, but retained their corrective effect on the coagulation of whole hemophilic blood. After the addition of washed suspensions of platelets, normal or hemophilic, the plasma regularly corrected the coagulation defect of hemophilic plasmas. The platelet suspensions themselves did not do so. It must be concluded, therefore, that the presence in one medium of both platelets and plasma was required for the production of whatever substance was involved in the reduction of hemophilic coagulation time. Brinkhaus prefers to consider that some plasma factor acts upon platelets to release contained thromboplastin. Quick (*Fed Proc 6 284, 1947*) believes that the platelets release something which activates a thromboplastin-precursor in the plasma. The normalcy of hemophilic platelets is indicated by both their investigations, and the occurrence of some defect in the plasma of hemophilia is now generally accepted. Whether the defect consists of absence of thromboplastin, absence of activator, absence of some unspecified globulin, or excess of heparin still remains to be unequivocally demonstrated.

S.E.

**LES HÉMATOMES INTRACRANIENS DES HÉMOPHILES (INTRACRANIAL HAEMORRHAGE IN HAEMOPHILIACS)**

*Paillard, J Boudouresque, J Tamalet* *Sem Hop Paris 24 432-436, 1948*

Only a few isolated cases of intracranial hemorrhage in hemophiliacs have been reported. The following 3 cases of this quite exceptional complication of hemophilia are thus of some interest.

The 3 cases are almost identical. After a minimum amount of trauma (a slight fall on to the head in two cases, and compression of the neck and jugular veins in the third), the downhill course was progressive after an initial symptomless period. In 2 of the cases, there were signs of progressive intracranial hypertension, with increasing headache, somnolence and papillary congestion, together with motor signs and aphasia. The diagnosis was clear. The great problem was treatment.

In all 3 cases, surgical intervention was necessary. Great subdural hematomata composed of liquid blood with numerous clots were found. All 3 patients died despite a transfusion one hour before operation. Intervention was inevitable in all 3 cases because of the onset of coma. The patients died, 1, 2, and 24 hours, respectively, after the operation. In none of the cases were thrombin, fibrin, gelatine or oxycellulose used.

It is to be regretted that the authors do not give more details of the quantity and number of transfusions, nor of the clotting time, information which would be of great value in ascertaining the exact cause of the operative failures.

J.P.S.

**QUANTITATIVE STUDIES ON THE COAGULATION DEFECT IN HEMOPHILIA** *A J Quick* From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee *Fed Proc 6 284, 1947*

The three commonly held theories of the pathogenesis of hemophilia include absence of some factor from hemophilic plasma, presence of some anticoagulant in hemophilic plasma, and poor breakdown of hemophilic platelets to initiate coagulation. Recent studies by Quick tend to reconcile, according to him, the differences between the first and the third of these theories.

Quick found that the serum obtained immediately after hemophilic blood has been allowed to coagulate contains unchanged fibrinogen and virtually as much prothrombin as the original blood. Incubation at 37°C produces coagulation of all the fibrinogen in one hour, but the prothrombin is unchanged (1 c.

only a small amount of thrombin is formed) Thromboplastin converts the prothrombin of hemophilic blood to thrombin stoichiometrically, not enzymatically Platelets, however, contain little thromboplastin, for when an extract of platelets is added to prothrombin in plasma, little conversion to thrombin occurs Normal plasma deprived of platelets by centrifugation shows a poor conversion of prothrombin to thrombin, but when such platelet-free plasma is added to hemophilic blood, the prothrombin of this blood is rapidly changed to thrombin and rapid clotting follows When normal platelet-free plasma is added to hemophilic plasma also low in platelets, little thrombin is formed

These data signify that thromboplastin may occur in plasma in an inactive form, and Quick believes that the platelets furnish an agent which enzymatically converts this inactive thromboplastin to an active form Hemophilic platelets behave normally, but hemophilic plasma, according to this work, lacks the inactive thromboplastin, and hence coagulates poorly Quick feels that the inactive thromboplastin is probably identical with Howell's plasma thromboplastin and with the anti-hemophilic globulin of the Harvard investigators The nature of the latter substance has not yet been chemically determined, but its identity with thromboplastin is an interesting, if undemonstrated, suggestion

S E

#### THROMBOPLASTIN IN THE URINE OF NORMAL AND HEMOPHILIC MEN *L M Tocantins and J N Lindquist*

From Jefferson Medical College, Philadelphia *Fed Proc* 6 215, 1947

Fresh human urine was found to contain thromboplastic substances which act by accelerating the conversion of prothrombin to thrombin Urine from hemophilic patients was found to have as high or even higher activity in this regard as urine from normal men The source of this material, and its nature, are not clear, but the authors suggest that it may come from the kidney itself, or may be the product of disintegrated tissue or blood cells cleared from the blood by the kidneys

S E

#### THE PROTECTIVE ACTION OF RUTIN AGAINST CAPILLARY INJURY *A M Ambrose and F DeEds* From the Pharmacology Division, Bureau of Agricultural and Industrial Chemistry, U S Department of Agriculture, Albany, California *Fed Proc* 6 306, 1947

These workers studied capillary permeability in rabbits by means of intravenous trypan blue injections the dye accumulates in areas of injury Capillary injury was produced in the rabbit's abdominal wall by various agents including chloroform, histamine, and negative pressure Untreated rabbits were compared with animals who had previously received rutin

In 22 rabbits to whom rutin had previously been administered, the appearance of trypan blue dye in chloroform-produced wheals was delayed as much as eight times above control, nonrutinized animals (Dosages and times are not given in this brief report) Rutin also prevented the appearance of dye after the application of negative pressure to the abdominal skin These are further data to be added to the rather meager experimental evidence supporting the clinical impression that rutin has a beneficial effect on capillary resistance

S E

#### BLOOD LEVELS OF CERTAIN CONSTITUENTS IN NORMAL ADULTS BEFORE AND AFTER INGESTION OF RUTIN *E*

*Papageorge and F Adair* From Emory University, Georgia *Fed Proc* 6 283, 1947

Despite the vague clinical impression that rutin is useful in certain patients with excessive capillary permeability, little objective experimental evidence has been presented to confirm this feeling The authors placed 15 normal adults on a low ascorbic acid diet for four weeks, and then tested them during successive two week intervals with rutin in the absence of ascorbic acid, ascorbic acid without rutin, and both drugs together The dose of rutin was 100 mg daily, as was also that of ascorbic acid

Rutin was found to have no influence on the serum levels of cholesterol, calcium, sodium, or vitamin K Ascorbic acid levels, although done, were not reported, but they were said to suggest that rutin tends to maintain a high serum level of this substance The relationship of these findings in the normal individual, to that in the abnormal patient with forms of vascular purpura (in whom rutin may be of value), remains to be found There is still no agreement on the mode of action of rutin, and little beyond an impression as to whether it is even efficacious

S E

SOME FACTORS INFLUENCING THE STRUCTURE AND RATE OF FORMATION OF FIBRIN CLOTS *J D Ferry, J T Fdsall, P R Morrison, V Kmet, and W F Leiter* From the Department of Physical Chemistry, Harvard Medical School, Boston Fed Proc 6 250-251, 1947

The authors studied fibrin formation in solutions of human fibrinogen and thrombin, in the absence of prothrombin and of fibrinolytic materials. They found that the initial rate of coagulation was linearly proportional to the concentrations of thrombin (within certain limits) and fibrinogen. By altering the pH, ionic strength, and chemical composition of the medium, they were able to produce various types of clot structures, including a clot which was coarse and readily contractile, and a clot which was translucent and friable.

S.E.

THE FINE STRUCTURE OF CLOTS FORMED FROM PURIFIED BOVINE FIBRINOGEN AND THROMBIN. A STUDY WITH THE ELECTRON MICROSCOPE *C V Z Haun and K R Porter* From the Department of Pathology, Harvard Medical School, Boston, Mass., and the Laboratories of the Rockefeller Institute for Medical Research, New York, N. Y. J. Exper. Med. 86 285-292, 1947

A technic is described for preparing clots from bovine fibrinogen and thrombin which are suitable for study with the electron microscope. The observations of this report are concerned chiefly with the effects of alterations of pH. It was found that clots are composed of meshworks of single and compound fibers, and that unit fibers have greater diameter and greater tendency for lateral association into compound fibers as the pH is decreased from 8.5 to 6.3. Cross striation of unit fibers was a striking feature of all clots studied. The periodicity of these striations was found to be constant (approximately 250 Å) and there was a precise coincidence of striations of unit fibers when these were associated side by side to form compound fibers.

The authors present a cautious discussion of the possible relationship between their observations and the supposed molecular structure of fibrinogen. Excellent photographs are included in the paper.

L.E.Y.

HEMOSTATIC PROPERTIES OF HEMOLYTIC AGENTS *M E Muhrer and A G Hogan* From the Department of Agricultural Chemistry, University of Missouri Fed Proc 6 280, 1947

This paper attempts to elucidate the increased coagulability of blood following certain hemolytic episodes. The authors, having found a sharp drop in coagulation time in hemophilic swine immediately after the injection of hemolyzed erythrocytes, determined that the same effect could be obtained by giving acetylphenylhydrazine, thioldiphenylamine, and other hemolytic agents. Analysis revealed that, simultaneous with the reduction in coagulation time, there was a sharp decrease in the fibrinogen, the cell volume, and the amount of prothrombin [sic]. For certain agents—notably saponin and sodium oleate—the fall in coagulation time was followed by a negative phase during which the coagulation time was increased above normal despite an increase in fibrinogen. This refractory phase was due to a further decrease in the amount of prothrombin which occurred with these agents.

It is not clear to what the reduction in coagulation time could be attributed. It should be pointed out that, clinically, transfusion reactions, which result in severe intravenous hemolysis, result in a hemorrhagic tendency, rather than the opposite, but presumably this is the result of damage to the endothelial lining of the vascular system.

S.E.

THROMBOPLASTIC FACTORS IN THE ESTIMATION OF PROTHROMBIN CONCENTRATION *C L Conley, W I Morris, II* From the Department of Medicine, Johns Hopkins University and Hospital, Baltimore, Maryland Am J M Sc 215 158-169, 1948

The purpose of this study was to determine, by Quick's method, whether prothrombin determinations in which different thromboplastin solutions were used gave comparable results. Rabbit foam emulsion prepared by the authors, two acceptable commercial thromboplastin preparations, and viper venom were employed. Very divergent results were obtained on human hypoprothrombic plasmas when saline dilution curves were used for reference. In dogs, the authors found that barium sulfate-treated plasma behaved more like that of dicumarolized (prothrombin-free) plasma than did plasma treated with aluminum.

hydroxide Even with human plasma prepared with barium, discrepancies between various thromboplastins persisted The authors point out the apparent complexity of the factors involved This again emphasizes the difficulty in quantitating the prothrombin test and in comparing results of various laboratories using different thromboplastin preparations

C A F

DICUMAROL POISONING *A J Draper, Jr* From Charlotte Memorial Hospital, North Carolina *J A M A* 136 171-172, 1948

This case report of a patient whose self medication with 8 or 10 ' tablets of dicumarol was followed by hematuria, bloody diarrhea, oral bleeding, and multiple ecchymoses, outlines a method of treatment for such cases of excessive dicumarolization The patient received 150 mg of vitamin K as synkayvite on the first day (120 intravenously in two doses, 30 mg intramuscularly), and 60 mg of synkayvite'' daily by vein on the second, fourth, and fifth day Response was prompt and sustained

S E

IDIOPATHIC THROMBOCYTOPENIC PURPURA A STUDY OF THREE CASES WITH SPECIAL REFERENCE TO CHANGES IN THE MEGAKARYOCYTES *E H Valentine* From Temple University Medical School and Hospital, Philadelphia, Pennsylvania *Am J M Sc* 214 260-267, 1947

Bone marrow studies of three cases of idiopathic thrombocytopenic purpura showed an increase in total number and a striking decrease in platelet forming in megakaryocytes These studies give further confirmation to the previous report of Limarzi and the careful morphologic studies of Dameshek and Miller, and emphasize the importance of sternal puncture in the diagnosis of this form of purpura

C A F

TWO CASES OF THROMBOCYTOPENIC PURPURA TREATED WITH FOLIC ACID *T H Gridley and T R Waugh* From the Department of Medicine of the Homeopathic Hospital of Montreal, (Quebec), and the Department of Pathology, McGill University *Canad M A J* 57 487-488, 1947

The first patient described was a 52 year old female who had had attacks of purpura for ten years She was found to have a platelet count of 3000 per cu mm, a prolonged bleeding time and positive Rumpel-Leede test Splenectomy was followed by a remission lasting only a few months Thrombocytopenic purpura again developed and persisted with varying severity for eighteen months The platelet count rose and bleeding ceased at this time following administration of 10 mg of folic acid per day This form of therapy was continued for an additional fourteen months without exacerbation of symptoms The results in this case of idiopathic thrombopenic purpura are considered sufficiently encouraging to justify further clinical trials

In the second case presented, thrombopenic purpura followed administration of bismuth Since use of this drug was apparently discontinued before folic acid was given, the favorable response described cannot be attributed to folic acid therapy

L E Y

STUDIES ON THE INTRAVASCULAR THROMBOPLASTIC EFFECT OF TISSUE SUSPENSIONS IN MICE I THE REACTION OF MICE TO INTRAVENOUS INJECTIONS OF A SEDIMENTABLE TISSUE COMPONENT II A FACTOR IN NORMAL RABBIT SERUM WHICH INHIBITS THE THROMBOPLASTIC EFFECT OF THE SEDIMENTABLE TISSUE COMPONENT *L Thomas* From the Department of Pediatrics, Johns Hopkins University Medical School and the Harriet Lane Home for Invalid Children, Baltimore, Maryland *Bull Johns Hopkins Hosp* 81 1-42, 1947

Intravenous injections of suspension of mouse brain tissue into mice were observed to produce generalized ataxia, clonic convulsions and coma This reaction was associated with cessation of capillary flow and formation of cell aggregates along with shortening of the coagulation time The coagulation time then became prolonged and the mice were resistant to toxic doses of the suspension The factor in tissue suspensions which produced the reaction could be sedimented at 12 000 r p m, a procedure which increased its heat stability The active factor was considered to be thromboplastin and its coagulating effect could be inhibited by heparin and congo red Incubation with normal rabbit serum was found to

inhibit the activity of the suspensions. The factor in normal serum producing the inhibition was found within the globulin fraction and was thermolabile at 60°C. A second factor, probably calcium alone, was shown to be necessary for the inhibitory activity of serum. These observations are of unusual interest in that they remove the study of blood coagulation from the highly artificial environment of the glass container to *in vivo* observations. Demonstration of the reversability of coagulation reaction *in vivo*, at least in its early phases, introduces a new concept.

R S E

ETUDE DE 114 CAS DE SYNDROMES HÉMORRAGIQUES EXAMINÉS EN L'ESPACE D'UN AN (ÉTUDE CRITIQUE DES ÉPREUVES FONCTIONNELLES DE L'HEMOSTASE) (STUDY OF 114 CASES WITH HEMORRHAGIC SYNDROMES EXAMINED OVER ONE YEAR [CRITICAL STUDY OF FUNCTIONAL TESTS OF HEMOSTASIS]) A Tzanck, J P Soulier and S Efros. From the National Blood-Transfusion Centre, Paris. *Rev Hémat* 2: 478, 1947.

The interest of this detailed study lies in the fact that all the 114 cases have been studied with the aid of the same technics over one year. The first part of the article is a critical study of these technics. The hemorrhagic syndromes are then classified according to the results of tests for hemostasis and the stage of hemostasis involved.

The classification was therefore as follows:

1. Involvement of the third, or plasma stage of hemostasis. These are the true disorders of coagulation (e.g. hemophilia, hypofibrinemia, hyporhombinemia, anti-coagulants).
2. Disorders of the second, or thrombocyte stage of hemostasis (e.g. thrombocytopenic purpuras and the very rare thrombasthenias).
3. The purpuras without thrombocytopenia but with abnormal results for one test (e.g. bleeding-time or capillary resistance).
4. Hemorrhagic syndromes in which all the tests for hemostasis are normal (e.g. allergic purpuras, dermatologic purpuras, and hemorrhagic syndromes without purpura).
5. Unclassifiable syndromes of the Banti type in which many hemostatic factors are involved.

The article ends with some therapeutic applications, in particular the necessity of studying the megakaryocytes in the marrow before every splenectomy, and the importance of neuro endocrine factors in the involvement of the first or 'parietal' stage of hemostasis.

J P S

FUNDAMENTAL WORKING CONCEPTS IN THE STUDY AND MANAGEMENT OF PATIENTS WITH ABNORMAL BLEEDING. L M Tocantins. From the Department of Medicine, Jefferson Medical College and Hospital, Philadelphia, Pa. *Amer Practitioner* 2: 479-485, 1948.

This paper elaborates on the thesis that abnormal bleeding results from an upset in the balance between hemostatic functions and forces exerting stresses on the vessels. The various extrinsic (physical and chemical trauma) and intrinsic (muscular contractions, blood pressure, etc.) forces which are often disregarded by the practitioner, are enumerated and their importance with respect to diagnosis and treatment is emphasized. The use of splints and elastic bandages in the management of bleeding in hemophiliacs is illustrated, and the necessity for minimizing surgical trauma in these patients is stressed.

L E Y

THE HEMORRHAGIC TENDENCY IN CONGESTIVE SPLENOMEGALY (BANTI'S SYNDROME). L M Tocantins. From the Division of Hematology, Department of Medicine, Jefferson Medical College and Hospital, Philadelphia, Pennsylvania. *J A M A* 136: 616-621, 1948.

This is a discussion of the etiologic factors of the hemorrhage in Banti's syndrome, based upon a study of 22 patients in whom the diagnosis was made. All 22 patients had various forms of hemorrhage, of which hematemesis and melena were most common (17 patients). It is pointed out that the spleen may shrink with acute hemorrhage, so that it is not palpable on admission of the patient to the hospital, but becomes palpable only several days (as long as ten days) later. The periodicity of episodes of hemorrhage is considered to be due to a cycle of (1) gradual increase in portal hypertension, which gives rise to (2) rupture of vessels (esophageal, gastric, hemorrhoidal), which results in (3) hemorrhage, giving (4) decompression, which ultimately gives rise to (5) another increase in portal tension, etc. The actual

causes of bleeding are (1) varices of the esophageal and rectal veins, with weakening of the vein walls, (2) thinning of the mucosa overlying these veins, (3) thrombocytopenia, (4) hypoprothrombinemia due to hepatic damage. Treatment in such cases includes the use of transfusions, vitamin K, bed rest, and pressure at the site of bleeding, if necessary. Splenectomy was performed in 9 of the 22 patients, with improvement and long survivals in most.

S E

**THROMBOPLASTIC PROPERTIES OF PENICILLIN AND STREPTOMYCIN** *D I Macht* From the Department of Pharmacology, Sinai Hospital, Baltimore, Maryland *Fed Proc* 6 160, 1947

Investigations of the effect of penicillin on the coagulation time are somewhat confused at the present time. Moldovsky and his co-workers (*Science* 102 38, 1945) first pointed out that the parenteral or oral administration of penicillin to normal subjects was followed by a marked fall in the coagulation time of the blood. The degree of reduction was inversely correlated with the level of penicillin in the blood, but the effect persisted after penicillin was no longer demonstrable in the blood. Hines and Kessler, on the other hand (*J A M A* 128 744, 1945) found that penicillin sometimes aggravated the effect of heparin (i.e., tended to increase the coagulation time) in certain patients.

Macht, in this report, found a reduction in the coagulation time of patients receiving parenteral penicillin (or streptomycin), but was unable to find such an effect in patients with hemophilia. Some 200 studies were therefore carried out on rabbits and cats, which showed a shortening of the coagulation time after the injection of penicillin. Penicillin acted within thirty minutes to cause a reduction of the coagulation time, and continued to act for several hours. Penicillin X was found the most potent in this regard, penicillin F, weakest, and penicillins K and G, intermediate. (See also Macht, *Science* 105 313-314, 1947.)

Clinical significance of these findings must await evaluation of these and contradictory results, especially those of Fleming and Fish (*Brit M J* 2 242, 1947). These authors found that a concentration of penicillin as little as 340 units per cc increased the coagulation time of blood, and 12,000 units per cc might result in incoagulability. They were led to their investigations by the finding that apicectomy, a dental procedure which requires good local coagulation of blood for its efficacy, was being hampered by lack of coagulation in patients who had had penicillin powder instilled into the wound. They suggest, therefore, that penicillin not be used locally if coagulation is important, but comment that the parenteral use of penicillin, which results in small unitage of the drug in the blood, has no effect on the coagulation time.

S E

**PURPURA AND INTUSSUSCEPTION** *H Wolfsohn* From the Fulham Hospital, London, England *Arch Dis Childhood* 22 242-247, 1947

The author reports the combination of Henoch-Schönlein purpura and intestinal intussusception in a 5 year old child. Four days before admission to the hospital, the patient suddenly developed migrating polyarthritides, involving successively the knee, the elbow and the wrist, and then complained of severe abdominal pain and purpuric spots on the buttocks and arms. On physical examination, in addition, a mass was felt in the right iliac fossa. Blood studies showed no thrombocytopenia, and bleeding time, coagulation time, and tourniquet tests were negative. At exploratory operation, an ileo-ileal intussusception was found and repaired. The boy subsequently recovered completely, except for a single attack of abdominal pain plus bloody stool six months after operation.

There are nineteen previous reports in the literature of intestinal intussusception in association with Henoch-Schönlein purpura. Sixteen of the total of 20 cases occurred in males, but both Henoch-Schönlein purpura itself, and intestinal intussusception without purpura, are more common in males than in females. Joint pains were present in 11 of 17 reported cases, and purpura was present in all 20.

The coincidence of the two conditions—intussusception and purpura—is too great to be accounted for by chance. The author considers explanations for this coincidence, and notes that it is probable that extravasation of blood occurs into the various coats of the small intestine, the resulting mass of submucosal blood favoring intussusception. Recovery from this complication occurred in 12 of the 20 cases in the literature, either spontaneously or following operative interference. The causes for the purpura

which is supposed to be allergenic, remain obscure, in this case, there was no evidence in family or patient for allergy

S.E

## LEUKOCYTES AND LEUKOCYTIC DISEASE

EXPERIMENTAL ATTEMPTS TO TRANSMIT INFECTIOUS MONONUCLEOSIS TO MAN *A S Evans* From the Section of Preventative Medicine, Yale University School of Medicine, New Haven, Conn Yale J Biol & Med 20 19-26, 1947

Twenty-one experiments with human volunteers were carried out in an attempt to transmit infectious mononucleosis by means of serum, whole blood and throat washings. Transitory symptoms consisting of pharyngitis, lymphadenopathy and lymphocytosis with atypical cells appeared in a few subjects, but in no instance did an unequivocal example of the disease develop. Failure to transmit the disease may be due to extreme lability of the agent or low degree of susceptibility of the 17 subjects. These results indicate that the refusal of some blood banks to accept individuals who have a past history of mononucleosis is overcautious.

R S E

INFECTIOUS MONONUCLEOSIS A REVIEW *B R Gendel and J E Cottrell* From the Medical Service, Veterans Administration Medical Teaching Group, Kennedy Hospital, Memphis 15, Tenn Am Practitioner 2 472-478, 1948

Nearly all of the important aspects of infectious mononucleosis are discussed briefly in this up-to-date review with well-chosen bibliography. Of particular interest are the references to jaundice, thrombocytopenia, recently reported autopsy and biopsy findings and experimental transmission of the disease.

L E Y

GRAVES ACCIDENTS DE SUFFOCATION DANS DEUX CAS DE MONONUCLEOSE INFECTIEUSE (SERIOUS COMPLICATION OF ASPHYXIA IN TWO CASES OF INFECTIOUS MONONUCLEOSIS) *A Lemierre, M Morn and M Almon* Bull et mém Soc méd d hôp de Paris, 51-54, Jan 16, 1948

The first case is that of a pseudo-phlegmonous throat infection. The finding of Klebs-Loeffler bacilli in the throat, led to the injection of 100,000 units of serum. The general condition was good, the temperature was 39.7°C. The blood count showed 5,620 00 red cells, and 21,000 white cells of which only 50 per cent were polynuclears. On the eighth day of the disease, the movements of respiration began to be obstructed both behind and below the sternum. Respiration which had been noisy for several days now became more difficult. The dyspnea increased the following day and the patient died suddenly on the ninth day of the disease. Pericardial puncture provided 20 cc of liquid which gave a positive Paul Bunnell reaction. No autopsy was performed.

The second case characterized by the severity of the pharyngeal signs, also caused one to think of diphtheria (the culture was negative). A diagnosis of infective mononucleosis was made from the blood count (16,000 leucocytes, of which 42 per cent were polymorphs) and the positive Paul Bunnell reaction. Laryngoscopic examination showed massive hypertrophy of the lingual tonsil which resembled that of the pharyngeal tonsil, and appeared to be responsible for the severe dyspnea.

On the seventh day, the dyspnea increased with obstruction both above and below the sternum. Tracheotomy was performed. The signs disappeared and the tube was removed on the twelfth day. Cure resulted.

The authors attribute the progressive dyspnea to the severity of the pharyngeal signs. It is possible that a neurologic factor plays a part and the torpor of the two patients was intense.

We think that it is interesting to note these observations which, added to the reported cases of rupture of the spleen, show that prognosis should be reserved despite the usual benignity of infective mononucleosis.

J P S

ACUTE INFECTIOUS LYMPHOCYTOSIS *S. Israel's* Winnipeg, Manitoba, Canada *Am J Dis Child* 74 722-724, 1947

The author gives a brief review of the literature on acute infectious lymphocytosis and reports the case of a 4 year old girl who appeared to be suffering from this disease. She had vomited, was drowsy, had enlarged tonsils and an injected pharynx, but spleen and lymph nodes were not palpable. The highest white blood cell count was 39,350 at which time there were 69 per cent small lymphocytes. Four days later when the lymphocyte count was the same, there were 8 per cent eosinophils, a finding also noted by others. Tibial marrow showed 22 per cent lymphocytes. Sheep cell agglutination test was negative. The spinal fluid was not examined.

There is an apparent need for studies on the etiologic agent of this disorder.

LEY

LA NEUTROPENIE FAMILIALE (FAMILIAL NEUTROPENIA) *J. Boussier and R. Neydt* Sang 18 521-529, 1947

The authors present a study of a very rare affection, observed by Gansslen in 1941 in 4 families.

They describe here 3 members of the same family with a permanent leukopenia of between 2,500 and 3,500, with a neutropenia of between 35 and 50 polymorphs, without modification of the Arneht count. The red cell and platelet counts (in the case in which these last were estimated) were normal. Nothing abnormal was found clinically, the general state of health was good and the condition was discovered by chance. These subjects did not seem to be particularly prone to infection.

In the case most completely studied by the authors, the myelogram showed 49 per cent of granular cells and 41 per cent of erythroblasts. This defect is transmitted as a dominant non-sex-linked character.

The authors discuss the differential diagnosis between this condition, the splenic neutropenia of Wisemann and Doan, the chronic sporadic neutropenias and Fanconi's disease.

One should add that there may be a connection also between this condition and the syndrome recently described by Estren and Dameshek (*Am J Dis Child* 73 671, 1947).

JPS

CONVERSION OF ACUTE INTO CHRONIC LEUKEMIA *R. Isaacs* From the Hematology Laboratory, Michael Reese Hospital, Chicago *Fed Proc* 6 394, 1947

Six patients with acute leukemia (blasts in the peripheral blood) showed a change in the blood picture to that of chronic leukemia, plus an associated remission in the clinical picture, after the oral ingestion of crude tyrosinase. Details are not given, and no follow-up is reported, beyond the statement that the patients lived from five months to one year after the initial diagnosis of acute leukemia.

SE

CHANGES IN BLOOD LYMPHOCYTES IN NORMAL AND RESISTANT RATS FOLLOWING TRAUMATIC SHOCK *D. D. Munro and R. L. Noble* From the Research Institute of Endocrinology, McGill University *Fed Proc* 6 168-169, 1947

The production of traumatic shock in normal rats was found to be accompanied by a marked relative and absolute lymphocytopenia in the circulating blood. The greater the trauma, it was found, the greater the reduction in lymphocytes. In all cases which survived, however, the lymphocytes had returned to normal within forty-eight hours. When shock was so severe that death ensued, the most severe depression of lymphocytes occurred.

When, however, identical trauma was applied to trauma-resistant rats, it was found that the initial lymphocyte level was higher than in trauma-sensitive rats, the fall in lymphocytes less, and the recovery quicker. The authors believed, therefore, that the degree of resistance could be related to the lymphocyte level after trauma.

These are further studies in line with the general thesis that the lymphocytes and lymphocyte-producing areas of the body are important in the immune mechanisms of the body.

SE



STUDIES ON ADRENOCORTICAL FUNCTION IN RELATION TO LYMPHATIC LEUKEMIA *L. Levin* From the Department of Hematology, Michael Reese Hospital, Chicago Fed Proc 6 270, 1947

The author presents further evidence of a possible relationship between the adrenal cortex and lymphatic leukemia, in line with the studies of White and Dougherty of a reciprocal balance between adrenal cortex and lymphocyte-producing centers of the body. The present work attempts to discover the nature of the anatomic alterations in the adrenals of mice with lymphatic leukemia.

In Furth's AK strain of mice, lymphatic leukemia is associated with an increase in the size of the adrenal glands, but this occurs only in males (38 per cent increase) and not in females (5 per cent increase). Levin analyzed the adrenals, and found that in all mice, male and female, with lymphatic leukemia, the concentration of the cholesterol in the adrenal was lower after leukemia (2.64 per cent) than before leukemia develops (4.78 per cent). He found too that injection of pituitary adrenocorticotrophic hormone did not affect the course of the disease or the alterations in the adrenals, even though it was given from the time of transmission of leukemic cells. Attempts to study the excretion of 17-ketosteroids in clinical patients with lymphatic leukemia, in order to test their adrenal cortical activity, yielded equivocal results.

These results suggested that there might well be a relationship between the adrenal and lymphatic leukemia, although they did not prove such a relationship.

SE

THE POVERTY OF THE IMMUNOLOGICAL MECHANISM IN PATIENTS WITH HODGKIN'S DISEASE *I. N. Dubin*  
The Division of Pathology and Bacteriology, University of Tennessee College of Medicine, Memphis, Tennessee Ann Int Med 27 898-913, 1947

Records of 262 patients with Hodgkin's disease were reviewed for evidence of their ability to form antibodies. Only 1 of 38 patients showed a positive tuberculin in contrast to an expected frequency of 50 per cent. There was likewise a low incidence of positive serologic tests for syphilis and apparent inability to make antibodies against Brucella and typhoid vaccine.

The authors feel that this immunologic deficiency is attributable to the damage to the reticuloendothelial system by the disease, and that this explains the susceptibility of these patients to bacterial infection.

C A F

RELATION OF THE ADRENALS TO IMMUNITY *A. White* From the Department of Physiological Chemistry, Yale University, New Haven, Conn Bull New York Acad Med 24 26-31, 1948

The author presents a brief review of the newer concepts of hormonal factors concerned in immunity, a field in which he and his associates have made important contributions. Particular reference is made to the effects of adrenal cortical steroids in stimulating the development of phagocytic cells in lymphoid structures and in accelerating the release of gamma globulin from lymphocytes. It is further pointed out that these steroids may produce an elevation of antibody titre either in a hyperimmunized animal or in a previously immunized animal with no circulating antibody. Such observations suggest to the author that pituitary-adrenal cortical control of lymphocyte structure and function may play an important role in anamnestic responses and may explain why various unrelated stimuli produce these responses. It is acknowledged, however, that antibody production does not cease in adrenalectomized animals and that other recent experiments make it clear that much remains to be learned concerning the relation of adrenals and other endocrine glands to immunity.

L E Y

USE OF FOLIC ACID DERIVATIVES IN THE TREATMENT OF HUMAN LEUKEMIA *L. M. Meyer* From the Kings County Hospital, Brooklyn, New York Tr New York Acad Sc 10 99-102, 1948

This is one of a series of articles (in the same publication) comprising a symposium on the use of several derivatives of folic acid in neoplastic diseases. Other articles discuss the chemistry and synthesis of anti-folic acid compounds, and their employment in carcinoma, sarcoma, lymphoma, and Rosai

chicken sarcoma. The present report discusses experience with such compounds in chronic and acute leukemia, and multiple myeloma.

Several different compounds were used. Teropterin or pteroyl-diglutamyl-glutamic acid was used without effect on blood, bone marrow, liver, spleen, or lymph nodes, in 2 cases of multiple myeloma, 2 cases of chronic myelogenous leukemia, and 7 cases of chronic lymphatic leukemia. In an additional patient with acute (lymphoblastic) leukemia, however, there was a fall in the number of white cells and blasts in the peripheral blood, a reduction in the percentage of blasts in the marrow with a corresponding increase in mature lymphocytes, and some transitory clinical improvement. There was rapid relapse, however, and death.

Diapterin (pteroyldiglutamic acid) had a similar effect in one other patient with acute lymphoblastic leukemia.

Five further cases of acute leukemia were treated with two other derivatives of folic acid, which have anti-folic-acid activity in bioassays: pteroyl-aspartic acid and methyl-folic acid. Similar results were noted in all these cases, namely, a reduction in the total white count in the peripheral blood, a reduction in the percentage of blasts, and a tendency for similar changes within the bone marrow. All these effects were transient, although their importance is suggested by the author's comment that the possibility that their occurrence was spontaneous, rather than related to the medication, was, according to his experience, negligible.

Regardless of hematologic effect, all treated patients, both in this and the other series (carcinoma, sarcoma, myeloma), felt clinically much improved. It was suggested by Gellhorn, in a discussion of this paper, that this effect was not specific and might be related to the known feeling of well-being following the use of glutamic acid in mental disorders. This discussor emphasized, too, that the results reported were fragmentary and not necessarily attributable to the various drugs utilized. They are at least, however, suggestive, and further reports will prove of interest.

S E

## TRANSFUSION JAUNDICE

HOMOLOGOUS SERUM JAUNDICE IN RECIPIENTS OF POOLED PLASMA. I. J. Brightman and R. F. Korns. From the New York State Department of Health, Albany, New York. *J. A. M. A.* 135: 268-272, 1948.

The authors investigated the incidence of homologous serum jaundice in patients who were given plasma for therapeutic purposes, and the incidence of plasma transfusions in patients whose death was wholly or partly attributed to hepatitis. No attempt was made to make a complete survey of each case, but the study was done in such a way as to give more or less minimum incidence value. The site of study was New York State, exclusive of New York City, and the time, apparently, was 1946-7.

Of 649 patients who received dried pooled plasma, hepatitis subsequently occurred in 29 (4.5 per cent). A statistically significant variation occurred with age: 8.7 per cent of 252 patients over 50 developed hepatitis, 2.2 per cent of 319 aged 20 to 50, and none of 78 aged under 20. No jaundice occurred in 1,597 household contacts of the recipients of the plasma, including 56 contacts of jaundiced patients. Calculated on the basis of an estimated 15,000 persons receiving plasma per year in the area involved, a yearly figure of some 675 cases of hepatitis might be anticipated. It was impossible to determine which lots of plasma might have been responsible, although a minimum figure of 4.7 per cent of all the lots used was calculated to have been icterogenic.

Of 51 deaths due to acute hepatitis, 15 patients (30 per cent) had received transfusions in the six months preceding death. Of these, 12 had received only plasma, 1 had received both plasma and blood, and only 2 had received only blood. American Red Cross dried pooled plasma was used in these cases as well as in the entire study.

Granted incompleteness of the investigation, the results presented seem to give minimum values for icterogenicity of pooled-plasma lots. The authors suggest two methods of dealing with the situation: (1) ultraviolet irradiation of plasma as part of its processing, and (2) substitution of albumin which can be heated to destroy the virus before distribution, for whole plasma.

S E

# NEWS AND VIEWS

## BLOOD CLUB

The first meeting of the newly organized BLOOD CLUB was held at Atlantic City on the evening of May 2, 1948, just prior to the meetings of the American Society for Clinical Investigation and the Association of the American Physicians. The meeting was organized by a committee consisting of William Dameshek (Boston), Carl V. Moore (St. Louis), and Maxwell M. Wintrobe (Salt Lake City). One hundred and eighteen were present at the dinner and another 25 or 30 appeared for the after-dinner discussions. Two subjects were taken up: (1) newer aspects in the pathogenesis of pernicious anemia, and (2) hemolytic mechanisms. The first subject was in the form of a round table discussion with the following panel: Maxwell M. Wintrobe, Moderator, Tom Spies, William B. Castle, Frank H. Bethell, Carl V. Moore, W. H. Sebrell, Joseph M. Ross, W. Jacobson (Cambridge, England), Robert W. Heinle, George E. Cartwright, and Thomas Jukes.

The panel was asked to discuss the following questions: Do patients with pernicious anemia respond to heptaglutamate or to the Di- and Tri-compounds? Does PGA do anything in sprue that liver extract does not? What is the present status of Wills Factor? What is the present status of experimental anemias designed to elucidate the pathogenesis of pernicious anemia and related disorders? How does one explain the role of PGA and liver extract in these conditions? What is the significance of the inhibitors? How is the action of thymine explained? What is the significance of vitamin B<sub>12</sub>? What is the relation of PGA to the neurological changes in PA and to glutamic acid metabolism?

Much of the discussion centered around the physiologic activity of folic acid and its conjugates and the possible relationship of these substances to liver extract. Drs. Heinle and Bethell emphasized the rather confusing experimental results in patients with the use of folic acid conjugates. Drs. Wintrobe and Cartwright discussed the production of megaloblastic macrocytic anemia in swine with the use of folic acid inhibitors. Good results were obtained with folic acid in contrast to the negative results obtained with liver extract.

Dr. Sebrell discussed his work with folic acid in experimental anemia and leukopenia. Dr. Ross discussed the neurologic lesions developing in cases of pernicious anemia following long continued use of folic acid as the sole medication. He pointed out that the glutamic acid in the folic acid molecule might be deleterious to the central nervous system tissue. However, no definite evidence for this effect was brought forward, although some work on brain tissue slices appeared to confirm these observations. On the other hand, Dr. Jukes of the Lederle Laboratories presented data indicating that folic acid, when allowed to act on brain slices in the Warburg apparatus, showed no effect on brain tissue. Dr. Spies mentioned his work with thymine and suggested the concept that pernicious anemia was in all probability a disorder of nuclear metabolism, especially since thymine was a product of nucleic acid degradation.

Dr Castle pointed out that the introduction of folic acid and the work with the various folic acid conjugates had resulted in considerable confusion as to the etiologic picture of pernicious anemia. This was heartily agreed upon by various other speakers.

The subject of hemolytic mechanisms was discussed in a more orthodox fashion, three speakers taking up different points of view regarding the mechanisms involved in excessive blood destruction. Dr William B. Castle (Boston) discussed the mechanical aspects of hemolysis with particular reference to the mechanical fragility of the red blood cell. He pointed out that agglutinins and other abnormal substances might result in increased mechanical fragility of the red blood cells, an important physiopathologic mechanism in red cell destruction. Dr Lawrence E. Young (Rochester, N. Y.) described some of his recent work with immune antibodies in hemolytic anemia and the production of auto- and iso-agglutinins in dogs. Dr William Dameshek (Boston) discussed abnormal agglutinins and their presence in acquired hemolytic anemia, the differentiation of acquired hemolytic anemia from the congenital types by serum antibody determinations, and the correlation of the presence of antibodies with a diminished survival time. He also pointed out that the antibodies of an abnormal type could be found in the hemolytic crisis of congenital hemolytic jaundice. In addition he presented observations of the reticulocytopenia and pancytopenia occurring in cases of hemolytic crisis, indicating possibly a hyperactivity of the spleen with resultant marrow inhibition and maturation arrest. The spleen in hemolytic anemia might have 3 functions: phagocytosis, inhibition, and antibody production. Observations in a recent case of hemolytic anemia associated with chronic lymphatic leukemia were cited, in which the picture of excessive blood destruction and of antibody formation were abruptly terminated following splenectomy, indicating that the spleen might well be a source of antibody production.

The meeting closed after 11 P. M. It was decided that the organization should be continued and that another meeting be held at approximately the same time next year. It was also decided that the group would continue on a very informal basis and that participation by anyone interested in the field of hematology would be invited. The round table type of discussion seemed to be in greatest favor with the audience, but it was decided that the exact type of program for the coming year would be decided upon by the committee.

#### MEETING OF FLORIDA ASSOCIATION OF BLOOD BANKS

A meeting of the Florida Association of Blood Banks was held in Miami, Florida on May 7-8, 1948. This meeting, sponsored by the Dade County Medical Association, was organized by Dr. John Elliott, Director of the Blood Bank and of the Medical Research Foundation of Dade County. A large number of workers in the field of blood and blood substitutes was invited to participate.

Dr. Alexander S. Wiener (Brooklyn, N. Y.) discussed the heredity of the Rh blood types and gave a clear exposition of his genetic theories and nomenclature in that field. Dr. Philip Levine (Raritan, N. J.) discussed the Rh factor with particular reference to the CDE nomenclature of Race and Fisher and disagreed sharply

with Dr Wiener's viewpoints Dr Ernest Witebsky (Buffalo, N Y) described his "9 drop test" for antibodies in the blood of the infant with erythroblastosis, and in addition made other observations regarding the use of antibody determinations in the mother for the detection of the presence or absence of erythroblastosis in the newborn infant Dr Louis K Diamond (Boston) described his technic for exchange transfusion in the treatment of erythroblastosis, pointing out the various advantages of the umbilical vein canalization method with the use of plastic catheters The mortality figures over a period of 20 years were impressive, indicating that with the newer technic, 90 per cent of infants with erythroblastosis may show complete recovery

Dr Virgil H Moon (Philadelphia) described his investigations in shock under various conditions and pointed out the fallacy of the term "lower nephron nephrosis" He advised the use of the term "tubular nephrosis" for the degenerative changes occurring in the kidneys under shock

Dr John Scudder (New York) described the technics in use in the Burn Clinic of the Presbyterian Hospital, pointing to the necessity for plasma and whole blood transfusions to maintain blood volume and blood counts He also emphasized the necessity for a "Burn Team"

Dr William Dameshek (Boston) gave a general discussion of anemia, its diagnosis and treatment, including recent investigations of the antibodies in association with hemolytic anemia Dr Louis Pillemer (Cleveland, Ohio) described his work in the characteristics and purification of plasma proteins and his contributions to the chemical nature of complement Dr Elmer de Gowin (Iowa City) described the use of whole blood and plasma products, giving a general discussion of transfusion technics and blood banking

Captain Lloyd Newhauser (United States Navy) discussed blood bank procedures in the event of an atomic bomb explosion when, for example, five bombs might be liberated over a large center of population

The final two papers were presented by Dr John Elliott who described indications for blood plasma and red cell transfusions, and by Dr Robert Elman (St Louis) who discussed the use of amino acids as a means of supplying parenteral protein

### AMERICAN BLOOD BANKS ASSOCIATION CONFERENCE

A meeting of the American Association of Blood Banks will be held Aug 26-28 in Buffalo, N Y, following a meeting of the International Hematology Society there The Association was organized in Dallas, Texas, Nov 19, 1947, to disseminate information relating to blood banking, to unite blood banks in times of disaster, to train personnel, and to promote similar services throughout the United States and its territories Membership is of two classes institutional, available to non profit, independent banks including those operated by A M A registered hospitals, and individual, available to any person interested in blood banking Requests for applications or information may be addressed to Miss Marjorie Saunders, LL B, Secretary, American Association of Blood Banks, 3301 Junius Street, Dallas

## IN MEMORIAM OF DR. HANS HIRSCHFELD

When I met Hans Hirschfeld for the first time, he was already numbered among the leading hematologists of the world. His rise had been difficult, since he was a pupil of Pappenheim, whose pithy criticism spared no one. Thus Hirschfeld had to obtain his place in the sun by fighting in the shadow cast by his great master, and he owes his reputation to his own scientific importance. It would be carrying coals to Newcastle to enumerate his numerous morphologic, clinical, and experimental works and try to appreciate their importance. He was much too modest and always harried by critical considerations, he disdained to lay stress upon his opinions, feeling sure that their correctness would be proved. He contended himself with expressing them, and he examined conscientiously every objection with which he was confronted.

When I went to see him in Berlin after my release from the Dachau Concentration Camp in 1940, I found him in back premises in a little room, almost squeezed in by his many books. He had been forced to quit his situation at the Cancer Institute of the Charité Hospital. He was allowed to attend only Jews, and had been eliminated from the editorship of the *Folia Haematologica*. Since the establishment of that journal by Pappenheim, Hirschfeld had labored for the perfection of that work under the greatest of sacrifices, and, after the shock of the first world war, had quickly regained for it its original international reputation. It was a grievous blow to him when his successor, devoted to national socialism, turned him away. Nevertheless, he uttered no word of complaint. Menaced most seriously in his existence, banished, the threatening cloud of the persecution of Jews ever harrying him and his family, Hirschfeld was an afflicted but by no means a broken man. No, he was a man who still took the liveliest interest in science and research. A presentiment that there would be no meeting again hovered over our last conversation and farewell. Afterwards we wrote one another several times. Then, the greetings on postal cards, signed only by an 'H,' failed to appear.

In the summer of 1945, a letter from Mrs. Hirschfeld informed me that her husband had died in the ill-famed Auschwitz Concentration Camp.

No tombstone adorns his grave. Even a last place of rest was begrudged him, who had worked so restlessly for the welfare of mankind. His works are a lasting monument to him. Those who knew him know what we lost by his death. He will ever remain a bright example to them of physician and man.

A. HITTMAIR, M.D.

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# BOOK REVIEW

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*La Maladie Hemolytique du Nouveau-Ne* (Hemolytic Disease of the Newborn) By M BESSIS PARIS, Masson & Cie, 1947, 248 pp

The physical isolation imposed by the Nazi regime on Europe during the period of 1939-1945 resulted simultaneously in a barrier to the ready exchange of scientific ideas. This volume, then, is all the more remarkable in that within the short space of two years, its author was able to read the voluminous literature on the subject of the Rh factor, adapt its specialized technics in his laboratory, and carry out clinical and experimental research on several aspects of the problem of diagnosis, pathologic physiology and treatment of Rh incompatibility and to organize his experience into this excellent summary.

The book is fundamentally a critical summation of the currently available knowledge of the Rh factor and its etiologic role in hemolytic disease of the newborn. It has the excellent qualities of careful organization, a clear manner of exposition, completeness without being overburdened with unnecessary chaff, and careful scrutiny of the various theories purporting to explain the mechanism of action of Rh incompatibility. The opening chapter is devoted to a discussion of blood groups and red cell antigens in general. This is followed by a description of the clinical, hematologic and pathologic findings in hemolytic disease of the newborn, weakened somewhat by the occasionally poor quality of the photomicrographs used as illustrations. An interesting discussion of some of the fundamental physiopathologic mechanisms of this disease is then presented, in which the author details and discusses his own work in this field, and the didactic sections of the book conclude with a discussion of treatment. One of the most helpful sections is the appendix, in which the author details methods of interrogation, blood grouping and performance of various diagnostic serologic examinations.

This book was expressly written to acquaint French speaking physicians with the latest developments in the field of Rh incompatibility. However, it is so well compiled and critically presented that all other physicians who read French will profit by it.

JACOB NEBER

# BLOOD

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### INFECTIOUS MONONUCLEOSIS

By SIR HENRY TIDY

EPFIEFFER, a pediatrician of Vienna, gave in 1889 the first clear description of the disease which we are here discussing, under the title Drusenfieber or glandular fever. He recognized that it was infectious and occurred in epidemics, described accurately the course of the enlargement of the cervical glands in young children, though he denied that other glands were involved, and stated that the glands never suppurated and that the prognosis was uniformly favorable.

Other observers in Germany quickly reported epidemics. Spread of its knowledge was somewhat slow but Park West (1896) reported an epidemic in America and Dawson Williams (1897) in England. The disease appeared to be successfully launched, but the diagnosis in sporadic cases rested on rather indefinite clinical features. It soon became confused with septic infections, and rapidly fell into disfavor. By 1900, it was practically dead. No mention of it appears in the Medical History of World War I, although information subsequently collected proves that it was not uncommon. It remained in suspended animation until 1920.

It is remarkable that during a period of thirty years, covering rapid developments in the knowledge of hematology, there should have been no systematic examination of the blood in a condition characterized by enlargement of lymphatic glands and often by enlargement of the spleen. To this lack of observation there is one exception. J. E. Burns, in 1908, fully described the lymphocytosis in two epidemics which he diagnosed as glandular fever, and published a well-documented report. The communication was completely overlooked and I was unaware of its existence until it was quoted by Bernstein (1940) in his monograph. Priority for the recognition of infectious mononucleosis clearly belongs to Burns.

Nevertheless, the establishment of the hematologic features of the disease did not come for practical purposes until 1920. Sprunt and Evans, in November 1920, published their observations on transient mononucleosis recorded in a series of six young adults during the previous six years. They recognized that the condition was infectious, observed the general glandular enlargement and gave an accurate though brief description of the various types of cells in the blood. They were clearly unaware of the existence of Pfeiffer's glandular fever, and thought they had discovered a new disease—a very venial mistake—and named it 'infectious mononucleosis'.

Authoritative articles soon followed. Longcope, in 1922, described fully the clinical aspects. He noted the long febrile period which might occur, and suggested the possibility of encephalitis in one case. Downey and McKinlay, in 1923, gave a complete description of the cells, beautifully illustrated, to which nothing effective has been subsequently added.



Meanwhile, Morley and I, in June 1920, recognized the existence of a transient lymphocytosis in the case of a boy whom we diagnosed as suffering from glandular fever, and we reported it with other evidence at a meeting of the Royal Society of Medicine in December 1920. At that time we were unaware of the article by Sprunt and Evans, but our attention was called to it when our article was in type and we formed the opinion that it was the same entity. In the course of the next few years, I saw a number of epidemics in boys' schools in England, and also cases in adults.

The recognition of the identity of infectious mononucleosis and glandular fever was not immediate in America, though Longcope used both terms in 1922, but it was probably general by 1925. The material on which the observations were based in America was predominantly from college students about the ages of 18 to 24 years, while in England it was supplied by resident preparatory school boys of 8 to 14 years. Although any type of the disease may occur at any age, there are considerable differences, when numbers of individuals are involved, between the clinical manifestations in the two age groups, the glandular enlargement being more marked at the younger ages. These factors no doubt account for the difference in nomenclature, for the disease is invariably known as infectious mononucleosis in America while in Britain it is generally named glandular fever. On the continent of Europe, the disease in young boys is now frequently described as "Pfeiffer's glandular fever" and that in adults as infectious mononucleosis, the identity of its infection in the two groups being accepted.

Neither resident college life nor resident preparatory schools are a part of the educational system in Europe outside Britain, and the recognition on the continent of a recoverable mononucleosis (other than that of whooping cough) was based on a different type of material, garbed in entirely other guise from that presenting in America and England. Deussing, in 1918, reported a series of cases with transient absolute lymphocytosis under the title "Über diphtherieähnlicher Anginen mit lymphatischer Reaction" (Angina resembling diphtheria with a lymphatic reaction). But it was a communication by Schultz in 1922, to the Congress for Internal Medicine on 'Monozytenangina' which first attracted attention to the subject. Both communications were based on the same type of material, being in regard to patients admitted to a hospital for communicable diseases who had membranous tonsillitis in which no diphtheria bacilli were found and in whom recovery followed without the injection of antitoxin.

The continental authorities went astray from the start. They were obsessed with the idea that the development of the lymphocytosis was due to a constitutional peculiarity of the patient resulting in a 'lymphatic reaction' and that the same angina in other individuals would result in a polynucleosis. The possibility of infection was scarcely considered. Secondly, they embarked on a tedious and sterile dispute amongst themselves covering many years as to whether the cells were monocytes or lymphocytes. They failed to recognize that both types of cells were often present at the same time and that either type might predominate at different periods in the same patient. Not until Glanzmann's monograph in 1930 did they admit that monocytic angina was a manifestation of infectious mononucleosis and recognize the influence of an infective factor as opposed to a constitutional diathesis.

The definite differentiation of monocytic angina from diphtheria was a clinical advance of importance, and it is somewhat surprising that so little notice was taken of it in American and British literature. The identity of monocytic angina with infectious mononucleosis was accepted without discussion by the specialists on communicable diseases, but its existence was certainly not generally known to the profession in Britain, even to the commencement of World War II. A brief clinical description of this type follows.

Monocytic angina or the anginose type of infectious mononucleosis is characterized by the development of a tonsillar membrane or of ulceration. The membrane in typical cases is indistinguishable in appearance from that of diphtheria, although it is a true membrane, and it often forms very rapidly. Edema of the neck and tenderness of the enlarged cervical glands is common but suppuration is very rare. Edema of the fauces causes great discomfort and anxiety, but in spite of this and the high temperature, the patient does not appear to be severely toxic nor does he become so, although the membrane may persist for several days or more than a week before separating, after which the symptoms improve with surprising rapidity. There is a previous period of slight malaise for two or three weeks with increasing sore throat, and some glandular enlargement may or may not have been observed.

There is no proof that the disease is infectious at this stage or that it spreads in this form, although a number of cases may occur in a unit of young adults. It is possible that the angina is a complication connected with the leukopenia which is often present initially, before the mononucleosis develops.

An extensive epidemic in England in 1930 was characterized by the severity and long duration of the attacks and by the high proportion of adults involved. During an initial febrile period which might last several weeks, the constitutional symptoms in this type are often suggestive of typhoid, and the characteristic features of infectious mononucleosis absent. Glandular enlargement develops late and is rarely of any great extent. The blood at the onset may show a definite polynucleosis. In prolonged cases, this may be observed to subside and for a period the blood count may be strictly within normal limits or passing to a leukopenia. The mononucleosis tends to develop about the time of the glandular enlargement and with their appearance the constitutional symptoms improve, often very rapidly, and the patient becomes convalescent.

Sporadic cases of this type are not uncommon and one instance was included in Longcope's article in 1922.

Four groups of clinical manifestations have so far here been indicated (1) Pfeiffer's glandular fever in children, characterized by rapid and visible swelling of the cervical glands and a short duration, (2) infectious mononucleosis in young males with a longer but milder febrile stage and comparatively slight glandular swelling (3) monocytic angina and (4) long febrile types with late and slight enlargement of glands.

This grouping was useful while the clinical features of the disease were being carefully studied, but the disease is apparently a single entity and every permutation and combination of the four groups occur. Milder cases often fall fairly clearly into one group, but this is rarely so for the severer forms.

## THE BLOOD PICTURE

It is probable that mononucleosis develops in every case of infectious mononucleosis

All the blood-forming tissues are affected, myeloid, monocytic (or reticulo-endothelial) and lymphoid, but at different times and to different degrees and varying in different cases and indeed in the same case at different stages. The effect on one system may be decreasing while on another it is increasing, thus producing the rapid changes in the blood picture which is so characteristic of the disease. The sequence of the changes is best observed in the long severe febrile cases, but unfortunately the diagnosis is rarely made in the early stages.

The myeloid system is earliest involved, but less constantly or severely and for a shorter time than the other systems. In mild cases, there may be no change in the circulating myeloid cells, but in severer forms an initial polynucleosis, such as 15-20,000 leukocytes with 75 per cent polynuclears, is not infrequent. This initial polynucleosis is a common cause for the diagnosis being overlooked. Polynucleosis is always transient and initial and never develops during the course of the attack. The rise of the mononuclear reaction may overlap the fall of the polynuclear cells, but in the more severe forms this reaction is delayed and the blood count becomes within normal limits and may remain so for two or three weeks or more, or may fall further to a leukopenia before the mononucleosis appears. Leukopenia is fairly common at the onset or during the course of severe cases before the mononucleosis develops. It is mainly due to granulopenia but even the lymphocytes may fall. In milder clinical types a mononuclear reaction may be present at the first examination or within a few days of onset.

The monocytic and lymphocytic reactions overlap, but the monocytic system subsides first and a pure lymphocytosis is finally left. So rapidly may alterations take place in the types of white cells and their number and so great are the differences in different cases that no single blood picture is exclusively typical of the disease. But most characteristic during the active stages is the presence simultaneously of various types of mononuclear cells, particularly with a high incidence of monocytes, an appearance rarely seen in any other disorder of the blood.

## HETEROPHIL ANTIBODIES

Paul and Bunnell (1932) made the curious discovery that heterophil agglutinins develop in high titer in human serum in infectious mononucleosis and in no other disease, with some unimportant exceptions. The development of heterophil antibodies and the technic of estimation will not be discussed.

Important questions which arise are at what stage does the reaction become positive, and in what proportion of cases is it positive, and does a negative reaction exclude infectious mononucleosis?

In the common mild types the reaction is frequently positive at the first examination, which is usually four or five days after the onset. If the examination is earlier, the test may be negative or indefinite, the titer rising in the next few days. Owing to the certainty with which the diagnosis can be made on clinical and hematologic grounds, the test is not always repeated. Nevertheless, in these circumstances the reaction is positive in nearly 90 per cent.

But in the severer febrile forms the reaction may remain negative during several weeks of pyrexia and constitutional disturbances, and become positive about the same time as the mononucleosis and glandular swelling develop. It is striking how often the constitutional symptoms rapidly ameliorate within a few days of the rise in titer. Thus, the development of a positive reaction is related to the end of an attack rather than to the onset, and it may well be connected with the development of immunity, as Himsworth (1940) suggested. On more than one occasion, I have known the reaction to become positive for the first time during a relapse.

The titer has no constant relationship to the severity of the disease, the extent of the glandular swelling or to the degree of lymphocytosis. The time during which a reaction remains positive is very variable, but little more is known. The titer may fall from a very high dilution to negative in the course of a few days. In ordinary mild cases, it may become negative within two weeks of recognition, but it often persists for several weeks and has been found still positive after several months.

The significance of a negative test especially arises in epidemics in which all results are reported as negative, and in individual cases in which the test is repeatedly negative. The question arises whether or not there are two types of infectious mononucleosis, giving respectively positive and negative reactions. There is nothing inherently improbable in the existence of two viruses, but until we know more about heterophil agglutination, the evidence must be regarded as inconclusive.

I agree with the opinion of Paul and others that a positive reaction is proof of infectious mononucleosis and a negative reaction does not exclude it.

#### NEUROLOGIC MANIFESTATIONS

The neurologic manifestations of infectious mononucleosis have attracted attention recently and quite a number of cases have been recorded in the last few years. It is obvious that the presence of infectious mononucleosis in similar cases must previously have been overlooked. The existence of encephalitis was suspected by Longcope (1922) and by Glanzmann (1930) but the first clear descriptions of neurologic features were given by Epstein and Dameshek (1931) and by Johannsen (1931). The clinical pictures are extraordinarily varied and bizarre, and no two cases appear to be quite similar. The brain (encephalitis), meninges, cord, cranial nerves and peripheral nerves may be affected, either separately or in combinations or sequences. There is no constant order in which the ordinary manifestations of infectious mononucleosis and the neurologic symptoms respectively develop, or in their comparative severity. The glandular enlargement, lymphocytosis in the blood and in the cerebrospinal fluid, and meningeal or other neurologic symptoms may develop and subside simultaneously, as in Epstein and Dameshek's case. In other cases, the infectious mononucleosis may run its course and subside to be followed by nervous symptoms, lymphocytosis in the cerebrospinal fluid and a normal blood picture. Or again the symptoms of a benign lymphocytic meningitis may be subsiding before the features of infectious mononucleosis appear. In this last group the blood count may show an initial polynucleosis even with a high mononucleosis in the cerebrospinal fluid.

The symptoms of benign lymphocytic meningitis are exactly reproduced in certain of the cases. It is also noteworthy that in the more severe neurologic forms, the blood changes tend to be late and the glandular swelling slight as with other severe types of infectious mononucleosis.

The heterophil agglutinins have been estimated in all recorded cases since 1938 and the test always has been positive in the blood at some stage with the exception of two cases in sisters (Thelander and Shaw 1941), but it has never been positive in the cerebrospinal fluid. Observers might naturally hesitate to attribute to infectious mononucleosis neurologic symptoms with negative agglutination.

Recovery from neurologic manifestations takes place with extraordinary rapidity. A comatose and paralyzed patient with an extensor-plantar response may be apparently normal mentally and physically in three days. The question of encephalitis requires further observation. Severe headache is the commonest symptom in neurologic cases and is also an occasional complaint in ordinary types. Children and adolescents may take a surprisingly long time, six to twelve months, to recover their usual powers of concentration and application after a simple attack although apparently physically normal. It is possible that this is a sequel of encephalitis.

#### ASSOCIATION WITH JAUNDICE

Jaundice is now not uncommon at the onset or during the course of the severer forms but the association has become frequent only in the last ten or twelve years. It was not recorded in the epidemic in England in 1930, but in 1935 and 1936 many cases were observed in St. Thomas's Hospital (Tidy 1937) and it is now a recognized complication. When occurring at the onset, the jaundice is often of considerable severity and there is nothing to distinguish the clinical condition from an ordinary infective hepatitis. As the jaundice subsides, the pyrexia persists and the diagnosis of infectious mononucleosis often follows the discovery of lymphocytosis in a routine blood count or the observation of some glandular swelling, which occasionally is present at the onset.

Jaundice adds the symptoms of infective hepatitis to the symptoms of infectious mononucleosis but does not appear otherwise to affect the course. Jaundice is rarely severe when developing later in the illness. Whether the jaundice is due to a separate virus cannot at present be determined.

#### DIAGNOSIS

Possible errors in diagnosis are numerous and mistakes in practice are not uncommon, but with the transitory nature of ordinary attacks they usually settle themselves without important consequences. They will not be considered seriatim.

In the severer febrile forms, there may be no means of establishing the diagnosis for several weeks. This may also apply to onset with jaundice or with neurologic symptoms. The possibility of infectious mononucleosis as the essential factor in some cases of benign lymphocytic chorio-meningitis should be borne in mind. The blood changes should not cause difficulty in differentiation from leukemia when the patient is seen in the acute stage. In acute leukemia, the toxic symptoms are always severe.

An occasional but extremely difficult diagnosis may be caused by the rare slowly-progressive chronic lymphoid leukemia. In the earlier stages, there are periods of exacerbation with pyrexia and moderate glandular swelling. The lymphocytes for several years may be only at the upper limits of normal. They gradually creep up and the diagnosis, long suspected, slowly becomes confirmed. Diagnosis is especially difficult when it is asked for on a patient some months after a pyrexial attack with lymphocytosis considered to be infectious mononucleosis. Either lymphocytosis or glandular swelling may persist for several months after infectious mononucleosis, but if both features are present for six months the diagnosis must be considered to be in doubt, unless it has been fully established. I have watched three such cases, originally diagnosed doubtfully as infectious mononucleosis, gradually develop into fatal lymphoid leukemia or lymphosarcoma over periods of three to ten years.

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# THE PATHOLOGY OF INFECTIOUS MONONUCLEOSIS

By R. PHILIP CUSTER, M D, AND EDWARD B. SMITH, M D

INFECTIOUS mononucleosis is not so uniformly a benign self-limited disease as is generally regarded. Thomsen and Vimtrup<sup>23</sup> (1939) reported 6 fatalities in a series of 500 cases treated in the Blegdam Hospital in Copenhagen. Two of these were complicated by some other infection, but in 4 cases death occurred in uncomplicated infectious mononucleosis and was due in each instance to central respiratory paralysis. The 2 autopsies in this group were the first we found described in the literature. Jersild<sup>14</sup> performed an autopsy on a patient who had had angina with sepsis, but who died of myocarditis attributed to infectious mononucleosis. Rupture of the spleen proved fatal in the patient examined by Ziegler<sup>11</sup> and fatal in 4 of the 7 cases of ruptured spleens in infectious mononucleosis which we reported.<sup>29</sup> The autopsy findings in 2 cases of Guillain-Barré syndrome associated with infectious mononucleosis were described by Ricker et al.<sup>27</sup> in 1947.\*

Pathologic changes in the *spleen* following surgical removal for rupture have been reported by Atlee,<sup>2</sup> King,<sup>16</sup> Darley et al.,<sup>7</sup> Davis et al.,<sup>8</sup> Milne,<sup>21</sup> Vaughan et al.,<sup>18</sup> and Smith and Custer.<sup>29</sup> *Lymph nodes* removed during the active stages of the disease were described by Gall and Stout<sup>12</sup> who included a review of the literature up to 1940. Moeschlin<sup>24</sup> presented his findings on aspiration biopsies performed in 3 cases (puncture of lymph nodes in all 3, of sternum in 2, and of spleen in 1). The *bone marrow* was erroneously described by Freeman,<sup>11</sup> but Nordenson,<sup>25</sup> and Limarzi, Paul and Poncher<sup>17</sup> have given accurate accounts of the changes in this tissue. A biopsy of the *liver* performed at the time of splenectomy was illustrated by Davis et al.,<sup>8</sup> and aspiration biopsies have been discussed by Kilham and Steigman,<sup>15</sup> van Beek and Haex,<sup>37</sup> and Bang and Wanscher.<sup>3</sup>

The aggregate of the findings in this group of scattered case reports is in essential agreement with our observations in the following series, although with few exceptions the descriptions have been incomplete and the tissue changes inadequately illustrated. Moreover, the diagnostic value of histologic appearances in the lymphatic organs has usually been underestimated. The purpose of this paper is to present as nearly a complete pathologic picture of infectious mononucleosis as possible.

## MATERIAL

This study was based on the following material, most of which was observed while the authors were on duty at the Army Institute of Pathology during the recent war.

Autopsies  
Lymph node biopsies

9  
100 plus

From the Laboratories of the Presbyterian Hospital in Philadelphia, and the Army Institute of Pathology, Washington, D. C.

\* These are our Cases 1 and 2 which we retained in our series because Ricker et al. gave attention more particularly to the neurologic features.

Bone marrow aspirates	25 plus
Bone marrow biopsies	2
Extirpated ruptured spleens	3
Tonsillar tumor	1
Liver biopsy	1
Skin biopsies	2
Causes of death in the fatal cases were as follows	
Spontaneous rupture of spleen	4 (*)
Guillain-Barre syndrome	2
Nasopharyngeal hemorrhage	1
Laryngeal edema	1
Airplane accident (convalescent case)	1

### PATHOLOGIC FINDINGS

Repeated reference will be made to the atypical or abnormal lymphocyte, the so-called infectious mononucleosis cell. We described this cell in a previous paper<sup>29</sup> as follows: "When stained lightly with hematoxylin and eosin in thin sections, it varies from 12 to 15 microns in diameter, occasionally larger, and is round except when distorted by crowding. The cytoplasm is homogeneous and faintly acidophilic. The centrally or eccentrically placed nucleus is sharply delineated by a thin membrane which blends with the marginal chromatin particles, chromatin is irregularly distributed to lend a mottled appearance, and it occasionally forms angulated bars. Indentation and folding of the nuclei can be demonstrated in relatively few cells. It is virtually impossible to determine in the sectioned material whether a true nucleolus is present or not, nor could "fenestration" be evaluated. We regard these cells as atypical or abnormal lymphocytes, closely related to, if not identical with, those found in the peripheral blood."

Following common usage we have employed the terms "infiltration" and "infiltrate" with reference to the presence of normal and atypical lymphocytes in connective tissues and as perivascular collars, i.e., situations where they are not normally found. We regard the majority of these cells, however, as of local origin and probably derived from pre-existing cells of the reticulo-endothelial system.

*Hematopoietic System.* *Lymph nodes* were usually but not invariably enlarged and displayed a variety of histologic appearances. In some instances, follicles were well preserved (fig. 1) and frequently assumed rather striking proportions (this is probably a transient phase). The structure of the follicular centers was not significantly altered except for a scattering of abnormal lymphocytes, histiocytes often contained cellular debris, and mitotic figures were common findings. In nodes such as these, the sinus structures were well preserved and contained varying numbers of normal and abnormal lymphocytes (fig. 2). The medullary cords were richly cellular and the same lymphocyte admixture was noted here. In a lesser number of cases the architectural pattern of the node was blurred, follicles being inconspicuous or even absent, and the sinus tracery was obscured as result of lymphocytic and reticulo-endothelial hyperplasia (fig. 3). This change was occasionally so striking as to

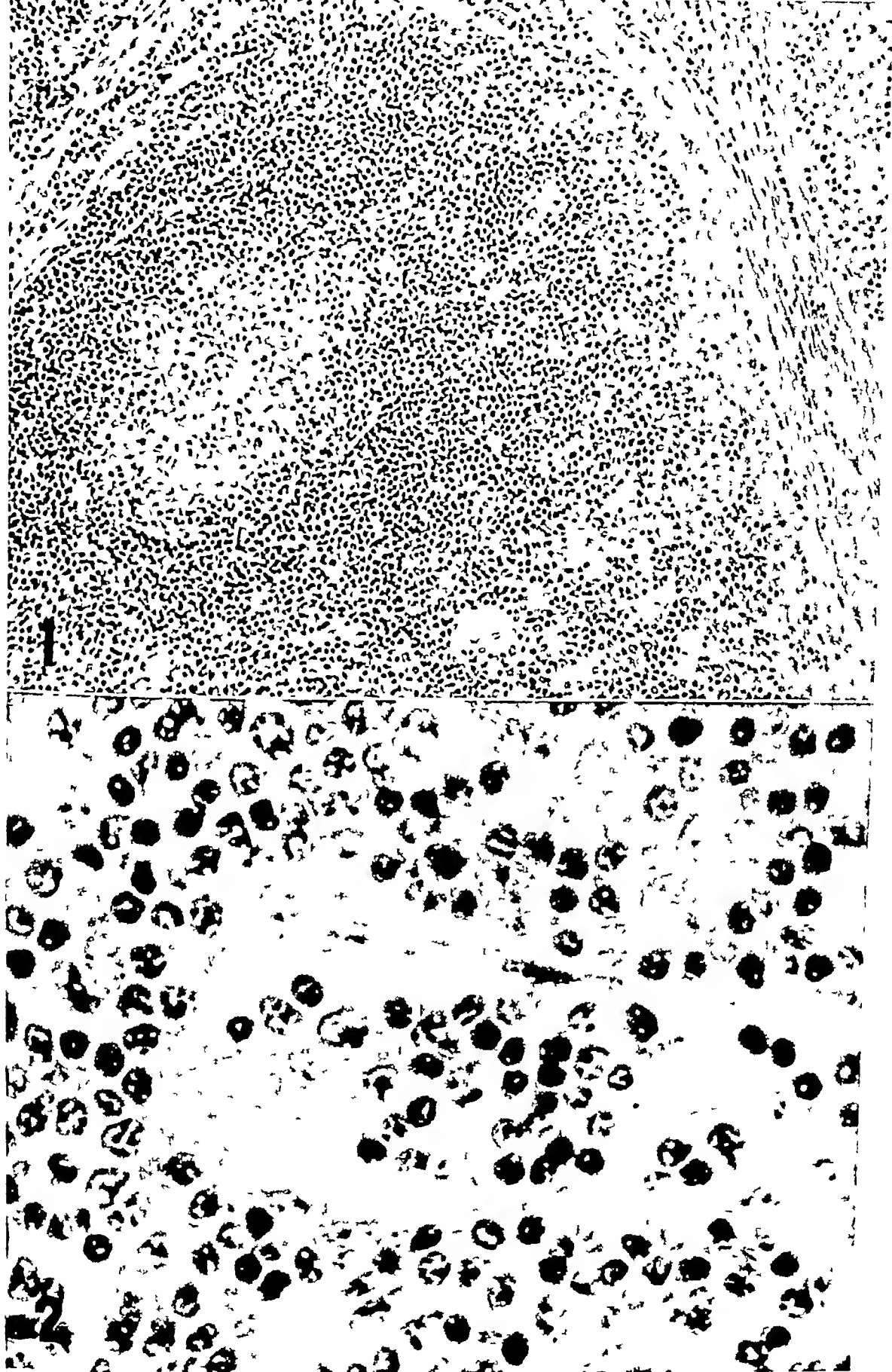
\* The immediate causes of death in these 4 cases were hemorrhage (2), postoperative pulmonary embolism (1), blood transfusion reaction (1). In 3 other cases, the ruptured spleen was removed and the patients recovered.



TABLE 1 — *Distribution of Lesions in Nine Fatal Cases of Infectious Mononucleosis*  
(+ distinct lesion, ± minimal lesion, — no lesion, blank, no tissue)

	Case No.								
	1	2	3	4	5	6	7	8	9
	AIP No								
	163849	151166	146828	67260	149279	151263	103388	94425	13146
Age	21	22	23	21	24	22	21	26	20
Sex	M	M	M	M	M	M	M	M	M
Color	W	W	W	W	W	W	W	W	W
Day of Disease	22+	17	30	14	?	13	33	35?	17
<i>Hematopoietic System</i>									
Lymph Nodes	+	+	+	+	+	+			+
Spleen (*)	+	+	+	+	+	+	+	+	+
Bone Marrow	—	—	—	—	—	—		—	
<i>Respiratory System</i>									
Paranasal Sinuses				±					
Pharynx (incl tonsils)	+					+			
Tracheo-bronchial Tree	+								
Lungs	+	+	±	±	—	±	±	±	+
<i>Cardiovascular System</i>									
Heart	—	+	+	+	+	+	±	+	
Aorta		+	—			—			
Peripheral Vessels	+	+	+	+	+	+	+	+	+
<i>Digestive System</i>									
G-I Tract									
Stomach	+		±	+	+				
Intestine	±	±	+		±				
Liver		+		+	+	+	+	±	+
Pancreas	—	—		—		+			
<i>Genitourinary System</i>									
Kidneys	+	+	+	+	—	+	+	—	
Lower Urinary Tract			+	+					
Prostate	+		+	+					
Testes	+		+						
<i>Endocrine System</i>									
Adrenal	+	+	±	—	±	+	+	+	
Thymus	±		±						
Pituitary		+	+	±	—				
<i>Nervous System</i>									
Meninges	±	+	+	±			—		
Brain	+	+	+	+	—		+		
Spinal Cord	+	±							
Peripheral Nerves	+	+							
<i>Musculature</i>		—	+						
<i>Integument</i>	(None from autopsies, two biopsies positive)								
Weight of Spleen (gm)	675	760	150	355	(45)	(35)	470	(45)	†
Weight of Liver (gm)	2100	2600	1650	†	†	†	†	2738	†

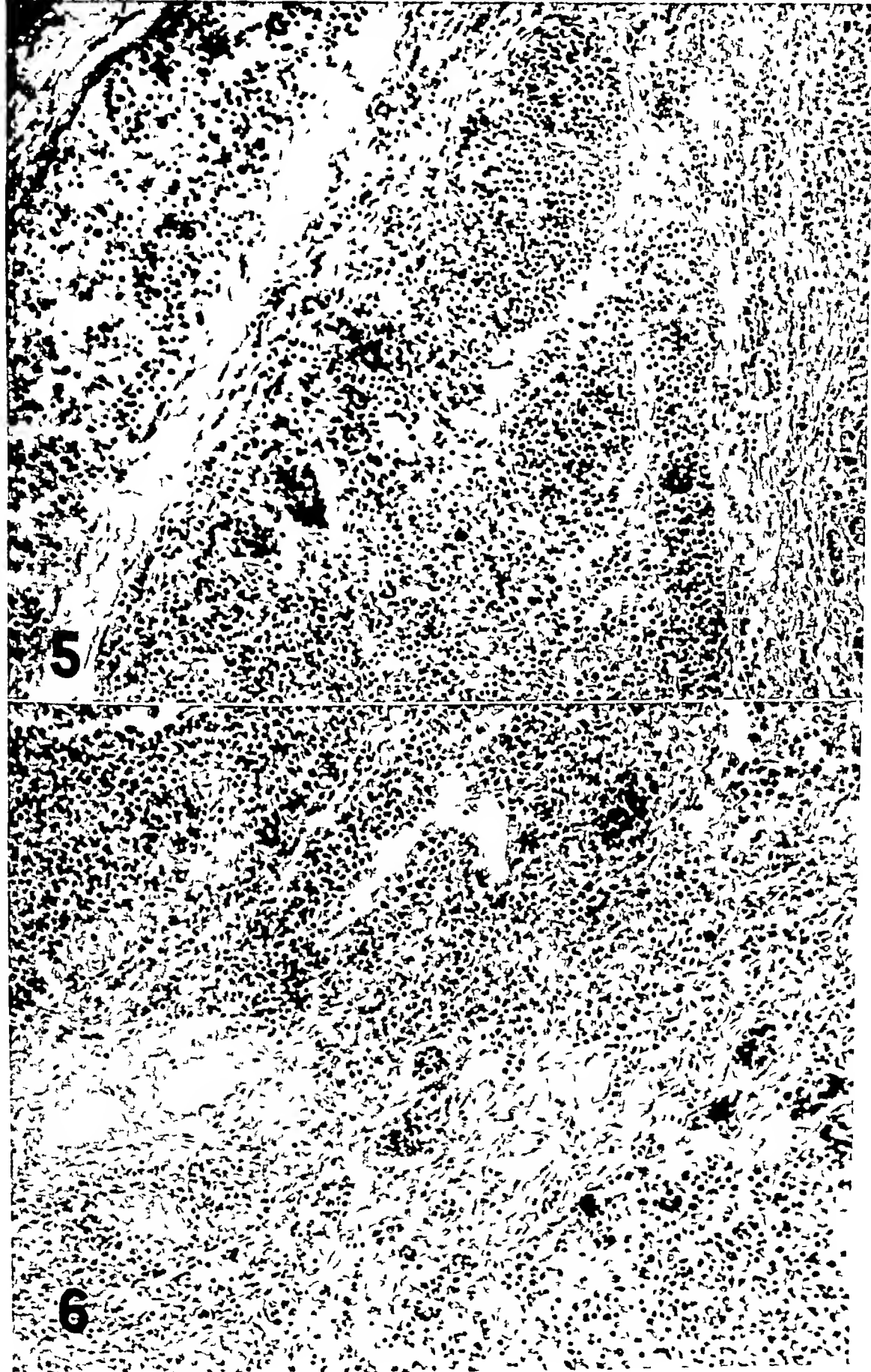
CAUSE OF DEATH Case 1, Respiratory paralysis (Guillain-Barre syndrome), Case 2, Respiratory paralysis (Guillain-Barre syndrome), Case 3, Airplane crash, Case 4, Hemorrhage from ruptured spleen, Case 5, Hemorrhage from ruptured spleen, Case 6, Edema of glottis, Case 7 Lower nephron syndrome (transfusion post splenectomy) Case 8, Pulmonary embolism (post splenectomy), Case 9, Hemorrhage



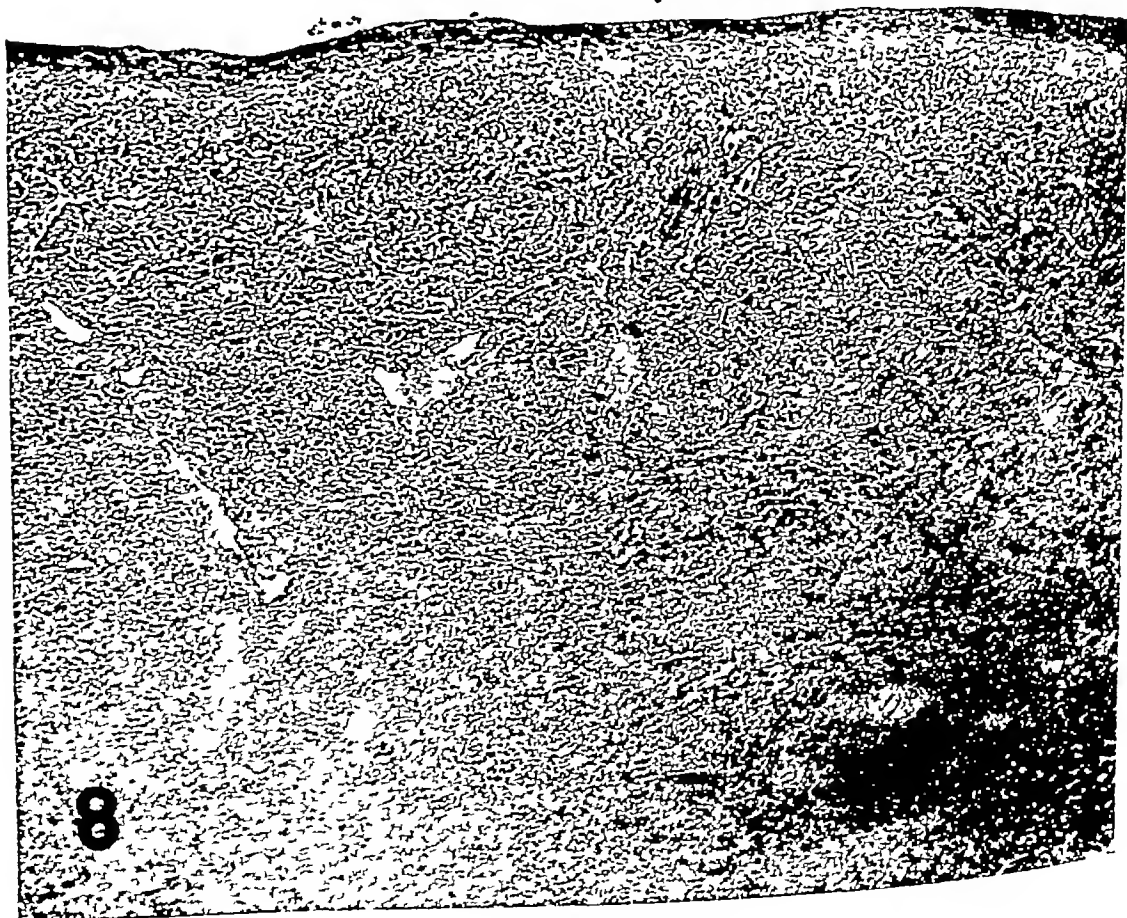
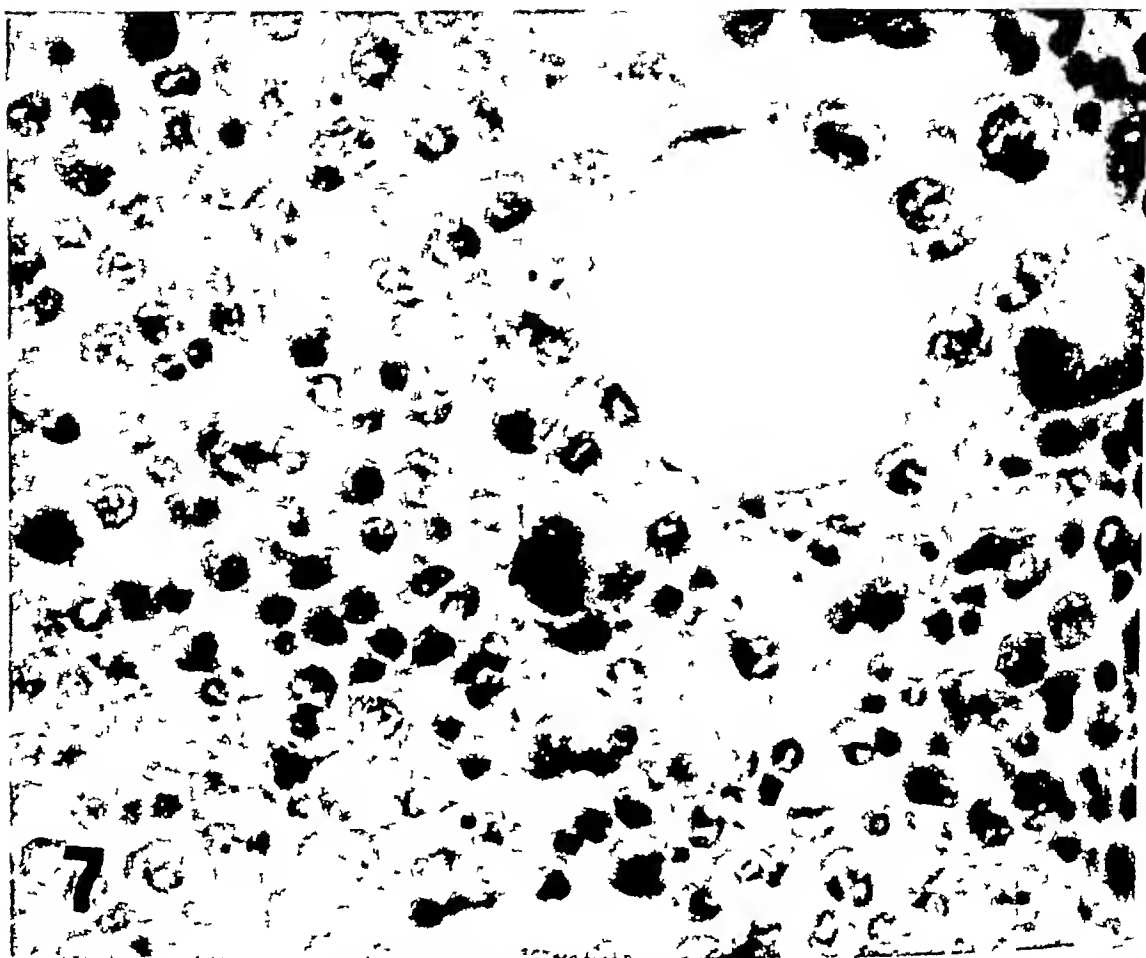
FIGS 1-2 (Sec p 846)



FIGS 3-4 (Sec p 846)



FIGS 5-6 (See p 846)

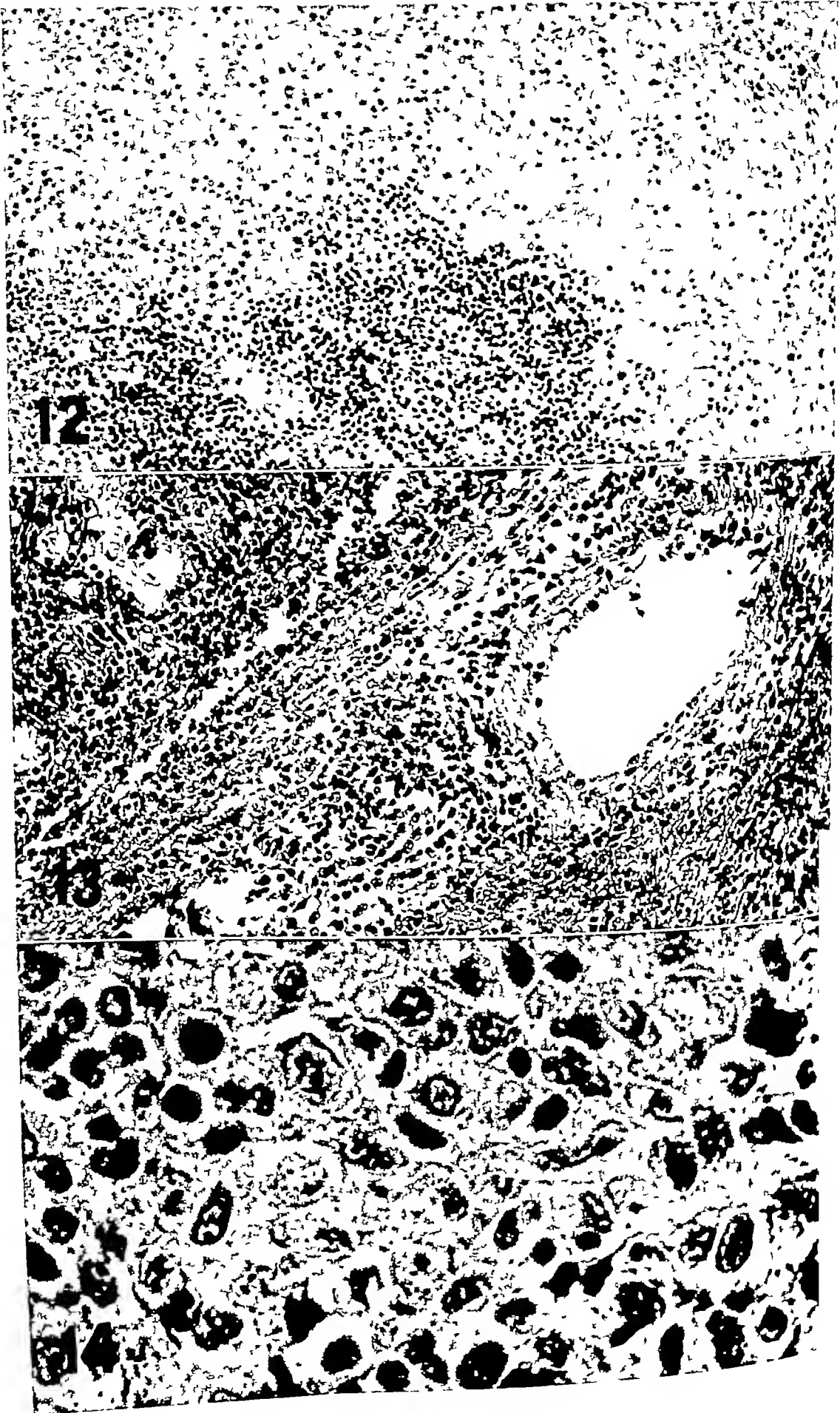


FIGS 7-8 (Sec p 846)

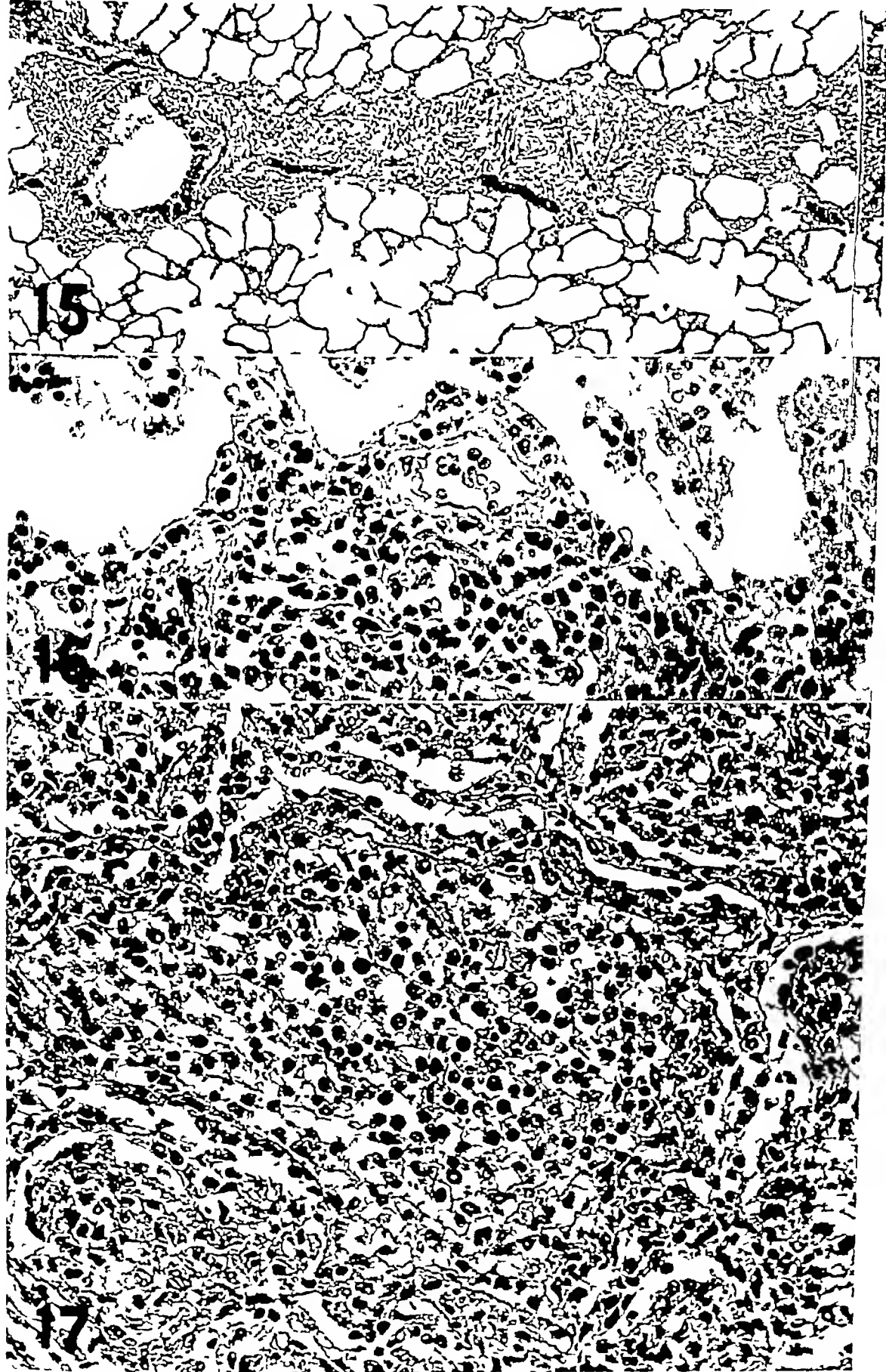


FIGS 9-11 (See p 846)



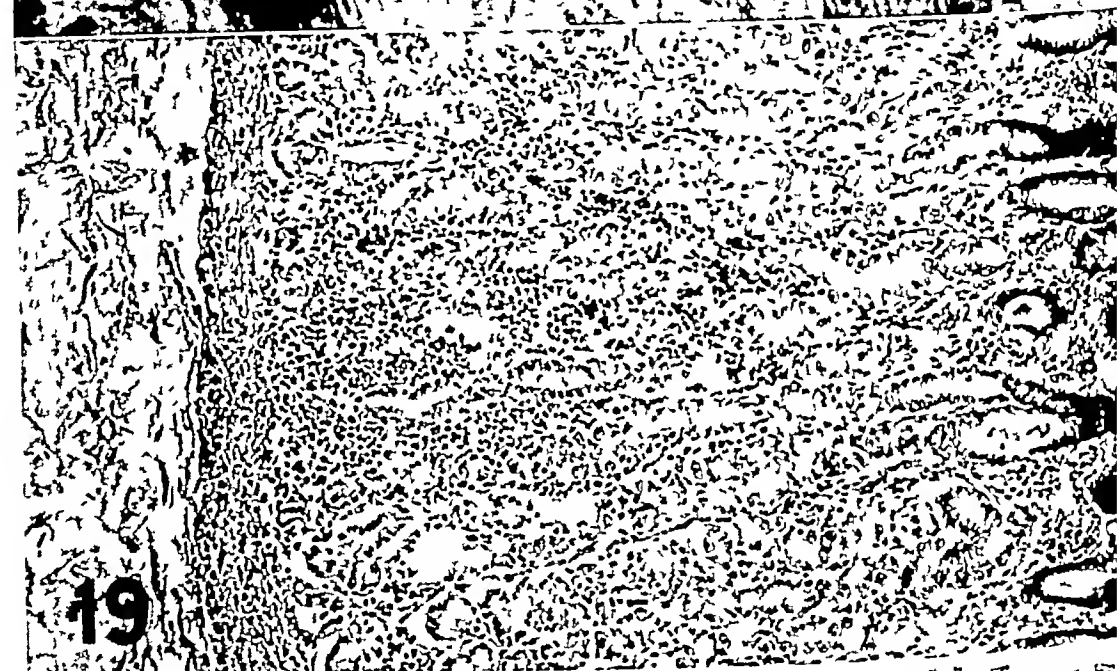
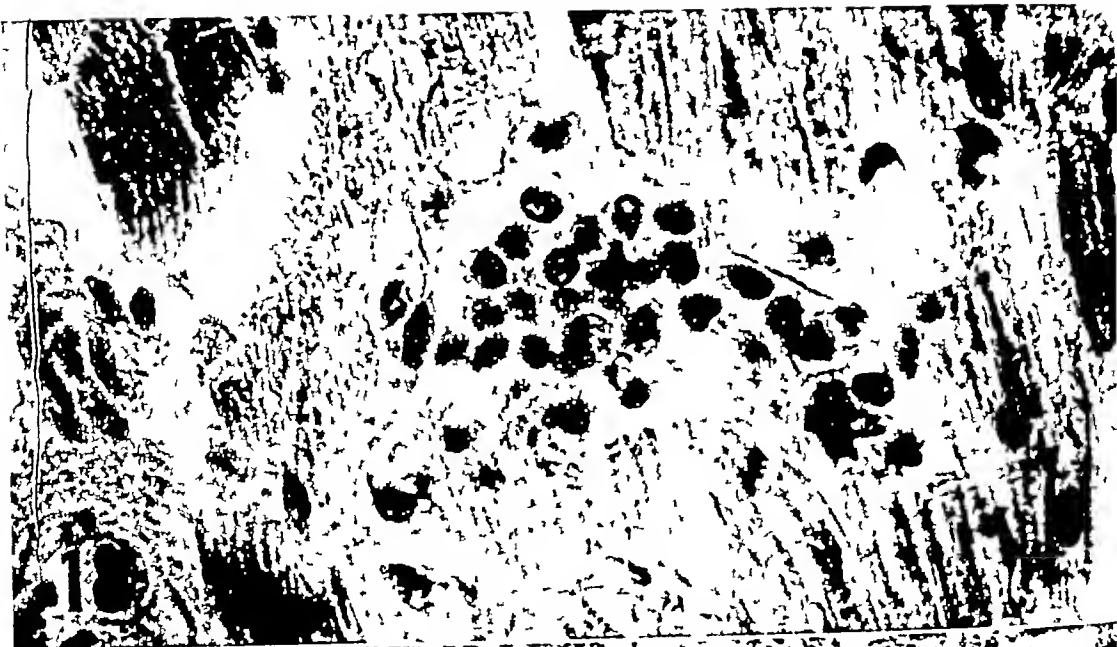


FIGS 12-14 (Sec p 846)



FIGS 15-17 (Sec p 846)

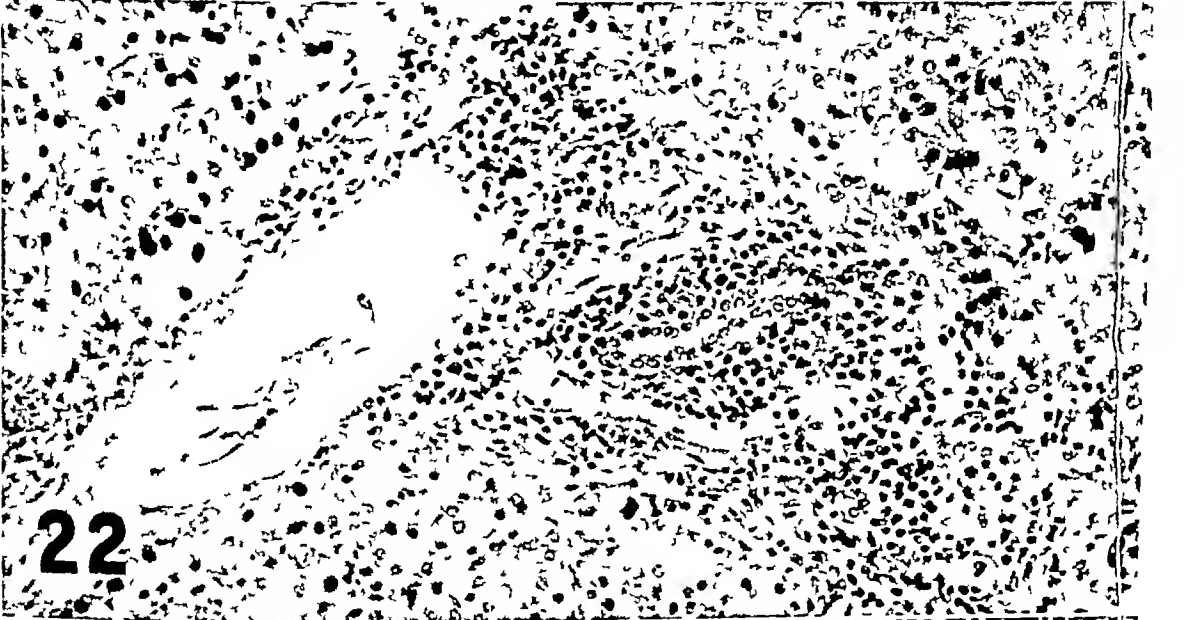




FIGS 18-20 (Sec p 847)



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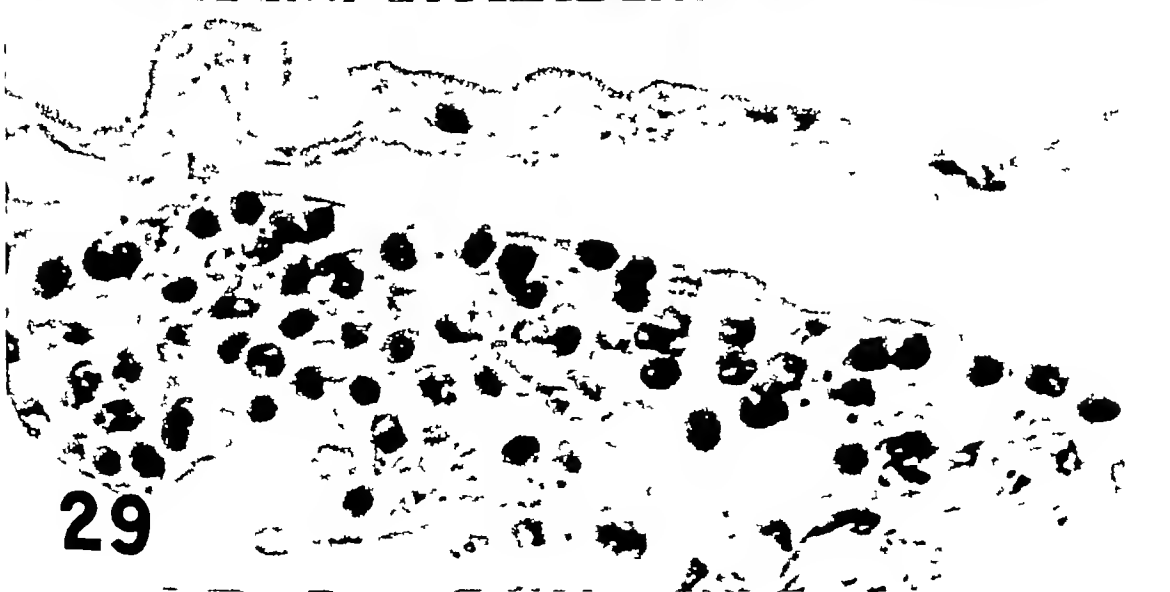
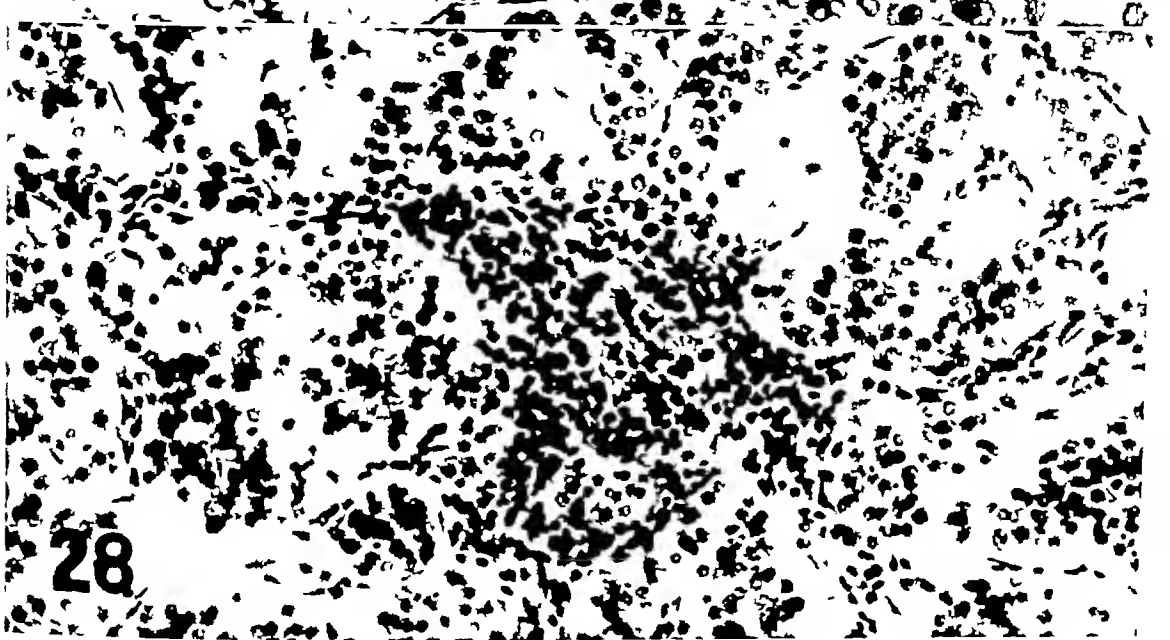
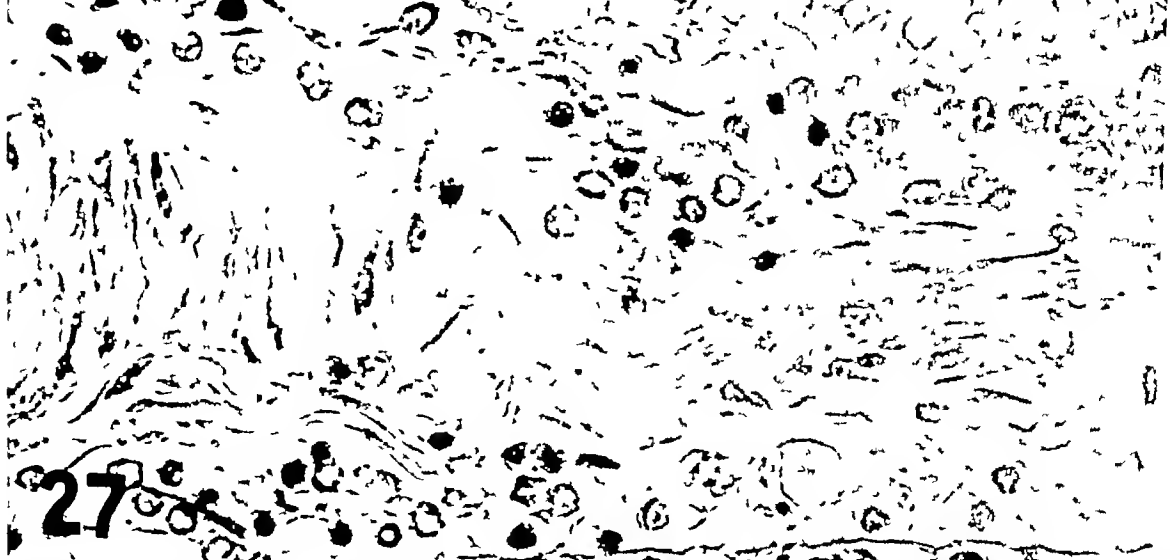
22



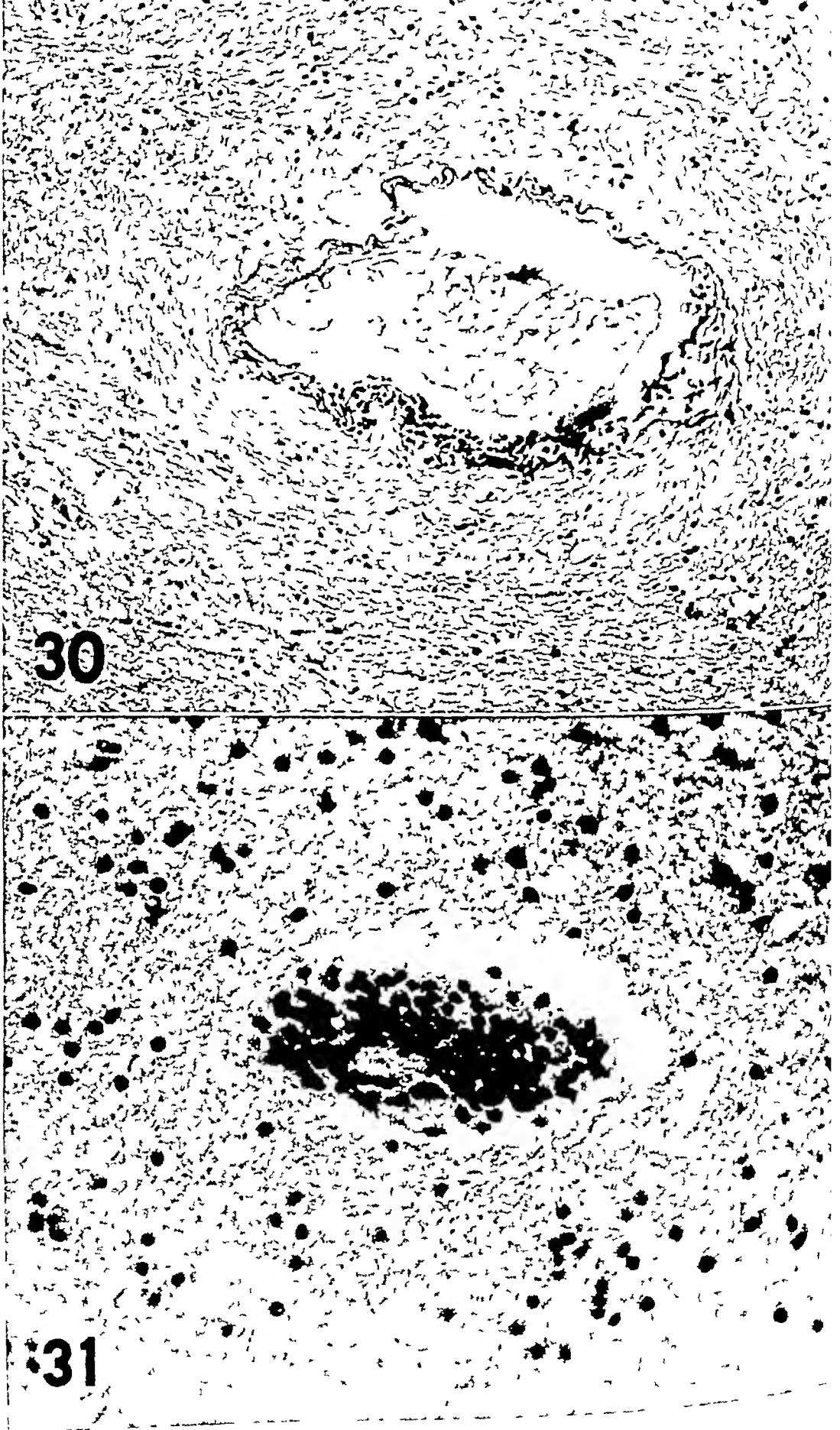
23



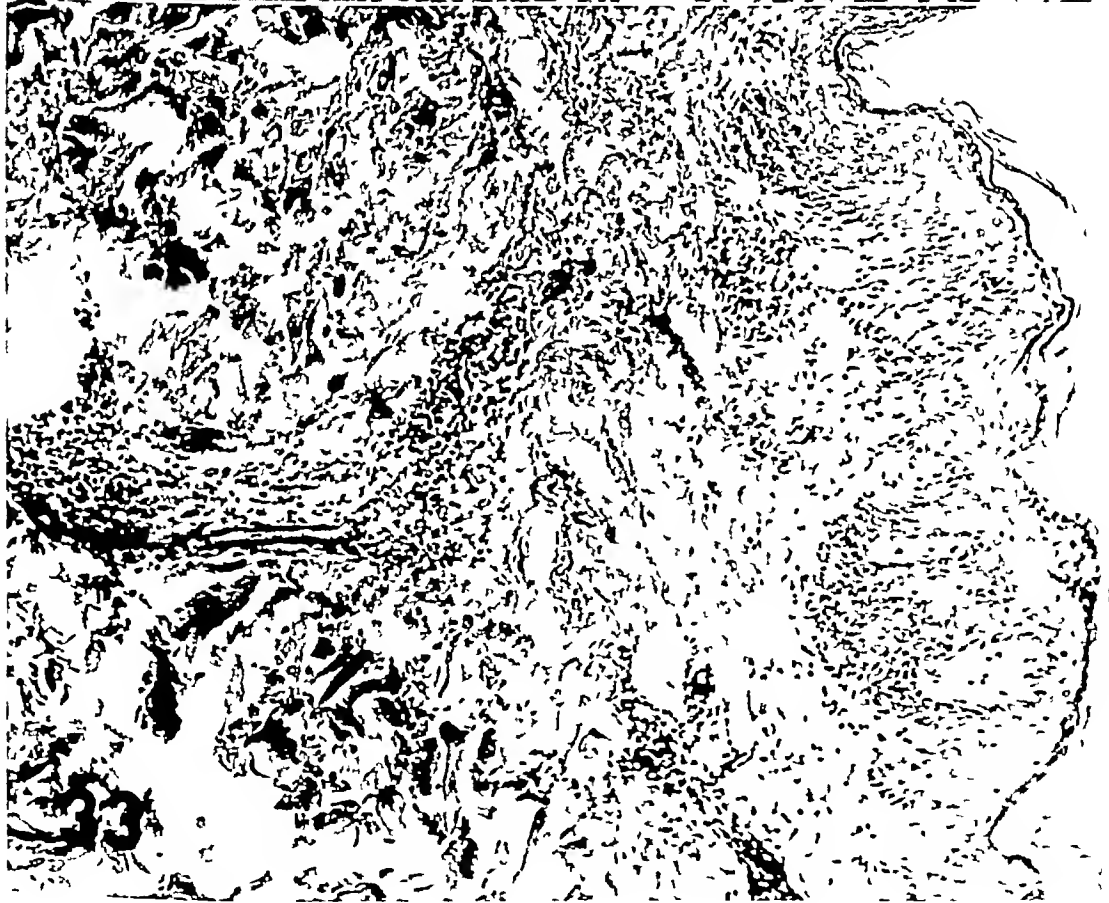
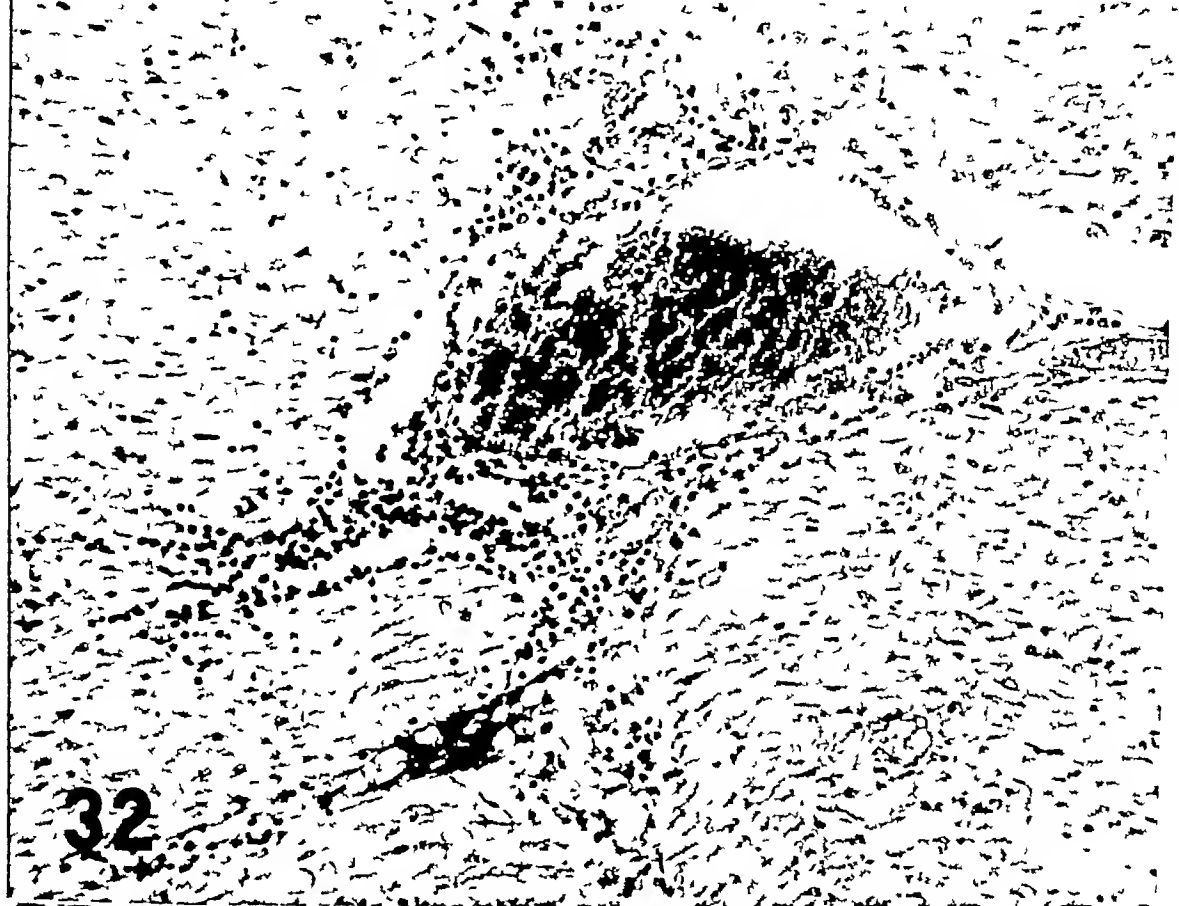
FIGS 24-26 (Sec p 847)



Figs 27-29 (Sec p 847)



FIGS 30-31 (See p 847)



FIGS 32-33 (See p 847)



FIG 1 LYMPH NODE A representative field from a node in which the general pattern was preserved. The follicle illustrated is rather poorly defined, but shows active proliferation in the follicular center and lymphocytic hyperplasia in the periphery. The sinusoid in the upper left is choked with normal and atypical lymphocytes. Reticulum cell outcropping is noted in the lower right, and the trabecula to the right displays a lymphocyte reaction ( $\times 200$ ).

FIG 2 LYMPH NODE A medullary cord at higher magnification presents hyperplasia of lymphocytes, reticulum cells, and lining endothelium of a sinus. The sinus contains normal and atypical lymphocytes, one of the latter lying outside of the sinus in the upper center ( $\times 1000$ ).

FIG 3 LYMPH NODE An extraordinary hyperplasia observed in a node removed during the peak of the illness. There is a striking hyperplasia of reticulum cells and abnormal lymphocytes to a degree that the entire nodal pattern is virtually obliterated to give the impression of a malignant lymphoma. The sinus tracery is preserved, however, marked by compact circumscribed aggregates of small lymphocytes ( $\times 200$ ).

FIG 4 LYMPH NODE Higher magnification of the node shown in Fig 3, emphasizing the resemblance to a malignant lymphoma of the reticulum cell type. True nucleoli are small and not so conspicuous as irregular chromatin clumps and angulated bars. Mitoses are plentiful, but never atypical ( $\times 1000$ ).

FIG 5 LYMPH NODE Section through periphery showing an afferent lymph vessel on the left, the lumen containing large numbers of normal and abnormal lymphocytes. The peripheral sinus of the node proper (right center) is also distended with round cells, and the capsule has virtually lost its identity by virtue of the lymphocyte reaction in the connective tissue, this is somewhat more marked than is usual ( $\times 200$ ).

FIG 6 LYMPH NODE Section through hilum disclosing increased cellularity of the interstices and packing of efferent lymph channels with lymphoid elements, even more marked than one finds in the capsule ( $\times 200$ ).

FIG 7 BONE MARROW There is a moderate granulocytic hyperplasia and a relative immaturity of the cells of this series. The small round cells shown in the picture are erythrocyte progenitors. Megakaryocytes are increased in number in proportion to the granulocytic hyperplasia. No lymphoid aggregate are present ( $\times 1000$ ).

FIG 8 SPLEEN View at low magnification demonstrating a blurred pattern except for prominent subcapsular blood sinuses, the sinuses usually contain clumps of normal and atypical lymphocytes. The capsule and trabeculae show dilution and partial dissolution by reason of lymphocytic infiltration. In the lower right is a small, poorly defined Malpighian follicle, in other cases the follicles are large with prominent germinal centers ( $\times 30$ ).

FIG 9 SPLEEN A stage in the dilution and dissolution of trabeculae as result of lymphocytic infiltration (presumably metaplasia of the trabecular connective tissue) ( $\times 200$ ).

FIG 10 SPLEEN Subendothelial lymphocytic zone in trabecular veins ( $\times 200$ ).

FIG 11 SPLEEN Adventitial lymphocytic reaction around intratrabecular artery ( $\times 200$ ).

FIG 12 TONSIL Extensive necrosis with small island of residual lymphoid tissue. Necrosis of this degree is infrequent and follows ulceration and secondary infection. Lack of neutrophil response is common but inconstant ( $\times 200$ ).

FIG 13 TONSIL Section through tonsillar bed discloses a marked lymphocytic reaction in the capsule (right) and in the interstices of an adjacent mucous gland (left) ( $\times 200$ ).

FIG 14 TONSIL Section through a rapidly developing unilateral tonsillar tumor occurring during the acute phase of infectious mononucleosis. As in the lymph node illustrated in Figs 3 and 4, the histologic appearances simulate those of a malignant lymphoma (heterophil culture 1260, complete recovery) ( $\times 1000$ ).

FIG 15 LUNG Peribronchial connective tissue and interlobular septum heavily infiltrated with lymphoid cells, the process involves interalveolar septa as well, rendering them thicker and more prominent ( $\times 30$ ).

FIG 16 LUNG Extensive interstitial pneumonitis with virtually no intra alveolar exudate, the cellular reaction is entirely lymphocytic ( $\times 450$ ).

FIG 17 LUNG Lobular pneumonia showing a rich fibrin network supporting a cellular exudate which is almost entirely of lymphoid type (In another case neutrophils predominated) ( $\times 450$ ).

FIG 18 HEART Focus of normal and atypical lymphocytes in the interstices of the heart, in most instances such collections are perivascular in position. Slight edema is occasionally noted, but muscle fibers show no structural changes ( $\times 800$ )

FIG 19 STOMACH An increased number of lymphocytes in the tunica propria. There is also a conspicuous zone of round cells on the deep margin of the muscularis mucosa ( $\times 100$ )

FIG 20 ILEUM Peyer's patches are moderately hyperplastic (one case) ( $\times 30$ )

FIG 21 LIVER Capsule displays a lymphoid reaction similar to that observed in the spleen. The connective tissue appears to be losing substance in proportion to the increase in lymphocytes ( $\times 350$ )

FIG 22 LIVER Minimal lymphocytic infiltration of the periportal connective tissue ( $\times 200$ )

FIG 23 LIVER Marked periportal lymphocytosis, maximal in our series, simulating lymphoid leukemia. Lymphocytes are also conspicuous in surrounding sinusoids ( $\times 125$ )

FIG 24 KIDNEY Lymphocyte accumulations in the interstitium of the cortex ( $\times 350$ )

FIG 25 KIDNEY Perivenous aggregates of lymphoid cells in the boundary zone ( $\times 200$ )

FIG 26 ADRENAL Perivenous collections of lymphocytes indistinguishable from those frequently encountered in any routine autopsy series ( $\times 350$ )

FIG 27 THYMUS Adventitial reaction around a small artery, cut tangentially, predominantly atypical lymphocytes ( $\times 750$ )

FIG 28 PITUITARY Rather extensive interstitial collections of lymphocytes ( $\times 350$ )

FIG 29 MENINGES Pia-arachnoid showing patchy round cell aggregates, chiefly in the neighborhood of blood vessels ( $\times 1000$ )

FIG 30 BRAIN Lymphocytic infiltration in the periphery of a moderate sized blood vessel. There is edema of the adjacent tissue ( $\times 200$ )

FIG 31 BRAIN Perivascular cuffing and edema resembling that seen in various proven virus encephalitides ( $\times 450$ )

FIG 32 ANTERIOR SPINAL NERVE ROOT Marked perivascular lymphocytic reaction in a case of infectious mononucleosis with Guillain-Barré syndrome. Appropriate stains demonstrated degeneration of myelin and distortion of axis cylinders of the nerve fibers ( $\times 200$ )

FIG 33 SKIN There is hyperemia and edema of the corium associated with round cell reaction about vessels and appendages ( $\times 100$ )

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simulate a malignant lymphoma (fig 4). Mitotic figures were abundant, but no atypical mitoses were observed. Connective tissue of the lymph node capsule, trabeculae, and hilum were more or less infiltrated with round cells, comprising for the most part both normal and atypical lymphocytes. The lymph sinuses in the hilum and perinodal tissue were usually crowded with similar cells (figs 5 and 6).

The spleen was invariably enlarged during the height of the disease as shown by the weights in our cases (table 1), the one normal weight of 150 Gm. in our series was in a convalescent patient who was accidentally killed. Milne<sup>21</sup> mentioned a 970 gram spleen which ruptured and was successfully removed. The histopathology of the spleen has been recorded in detail in a previous article.<sup>29</sup> To summarize briefly, the capsule showed varying degrees of infiltration with round cells, consisting largely of both normal and abnormal lymphocytes. The trabeculae displayed dilution of the fibromuscular structure by a similar infiltrate (fig 9), not infrequently to the point at which complete dissolution occurred, the site of the trabeculae in such instances was marked only by the trabecular vessels. Follicles were usually widely spaced, in some instances being well preserved and hyperplastic, in others existing merely as ill-defined aggregates of small lymphocytes (fig 8). Occasionally, the eccentric arteriole was the only landmark. The blood



sinuses of the spleen contained more nucleated cells than erythrocytes except when there was marked congestion of the organ. The nucleated forms again were mostly normal and atypical lymphocytes. The red pulp likewise contained a predominance of these cell types. The changes in the blood vessels were twofold: first, an adventitial infiltration of lymphoid elements around intratrabecular arteries (fig. 11), and second, a similar subendothelial infiltration of the veins (fig. 10). These vascular findings have been noted also in leukemias and scarlet fever, occasionally in chronic malaria and acute fulminating infections. The subintimal infiltrate in veins has also been noted in allergic states, especially drug sensitivity, in which the patient survived more than twenty-four hours.

The *bone marrow* contained no abnormal cells apart from those in the circulating blood (fig. 7). The marrows were either quite normal or in some instances moderately hyperplastic. Hyperplasia was usually limited to the granulocyte series, although megakaryocytes occasionally seemed more numerous than usual. Unfortunately in our Case 4, characterized by purpura and thrombocytopenia, no bone marrow sections were obtained.

*Respiratory System* Mucous membranes of the sphenoid and ethmoid sinuses were available for study in 1 case. These appeared normal apart from a minor submucosal round cell infiltration which was nonspecific in character.

The *tonsils* were examined in 2 of the autopsied cases. Both showed areas of necrosis and a reaction of normal and abnormal lymphocytes, with a variable admixture of plasmocytes and neutrophils (fig. 12). The round cell infiltration was also noted in the peritonsillar tissue (fig. 13). In 1 case, the lingual tonsils and posterior pharyngeal wall were sectioned and displayed a similar cellular reaction deep in the striated muscle and around mucous glands of the tongue and pharyngeal wall. The *larynx* and *trachea* in another showed a like mononuclear cell infiltrate. In a patient who recovered, a rapidly growing tonsillar mass was regarded as a tumor and removed surgically. The tissue presented an amazing proliferation of lymphoid and reticulo-endothelial elements to a degree mimicking reticulum cell sarcoma (fig. 14), the surface was ulcerated and the obvious inflammatory reaction was limited to the margin of the ulcer.

The *lungs* displayed a variety of appearances. In 2 instances, there was a frank pneumonic consolidation, the cellular exudate in 1 was of the classic polymorphonuclear neutrophil type, while in the other it was made up almost exclusively of large lymphoid cells (fig. 17). Most cases showed a more or less marked exaggeration of the peribronchial and bronchiolar collar, and in some the round cell reaction extended along the intra-alveolar septa to simulate interstitial pneumonitis (figs. 15 and 16). Clusters of lymphoid cells were also noted in the subpleural connective tissue.

*Cardiovascular System* In 6 of the 8 cases in which the *heart* was examined histologically, aggregates of lymphocytes were sparsely distributed within the myocardium in the periphery of small blood vessels (fig. 18). They were also present in small numbers beneath the endocardium. In 1 case, the reaction was virtually negligible, and in another completely absent in the tissue studied. The myocarditis and pericarditis were minimal and unassociated with any necrosis of muscle or

serosal reaction respectively, except in case 3 where the cellular infiltrate was rather prominent in some areas. No real pericarditis was encountered. The aorta was examined in 3 cases. It was quite normal in 2 patients who died respectively on the thirteenth and thirtieth day of the disease, but in the third, who lived seventeen days, perivascular cuffing was seen about the vasa vasorum in the adventitia, the cells being the usual normal and atypical lymphocyte varieties.

As regards the *peripheral vessels*, one may generalize that, apart from the lymphoid tissues proper, the lymphocytic proliferation was either adventitial or subintimal in location, irrespective of the organ or tissue examined. The arterial involvement tended to be adventitial, whereas the venous was subintimal.

*Digestive System* The tunica propria of the *stomach* contained collections of lymphoid cells considerably in excess of normal (fig. 19) in 3 of the 4 cases examined. In the fourth case, a convalescent patient who had been ill 30 days, the reaction was equivocal. Sections of *ileum* from 4 cases proved difficult to evaluate because of the variable prominence of lymphoid tissue under normal circumstances in this age group. The lymphoid proliferation was distinctly abnormal in only 1 of these cases (fig. 20) and the cellular reaction was that of the lymphoid tissues elsewhere.

*Liver* changes were uniform qualitatively in the specimens examined from 7 autopsies and 1 biopsy. The capsule in most instances showed varying degrees of involvement similar to that seen in the spleen (fig. 21). The lymphocytic infiltration was most pronounced in the periportal connective tissue. Quantitatively, this reaction varied from hardly more than the usual lymphocyte collar (fig. 22) to a degree approaching that of lymphatic leukemia (fig. 23), with lymphoid cell aggregates extending into the adjacent lobular parenchyma. In these latter instances, there was an apparent loss of some of the peripherally placed liver cells, associated with minor bile duct proliferation. Necrosis of the liver parenchyma was not observed in our series, except in 1 case where portal vein thrombosis followed splenectomy. There was no evidence of biliary obstruction. Those cases showing a lymphocytic leukocytosis also displayed considerable numbers of round cells throughout the lobule, but within blood sinusoids. This more diffuse intra-lobular cellularity was augmented by Kupffer cell hyperplasia.

Perivascular lymphocytic collars were noted in only 1 of the 4 cases in which sections of the *pancreas* were available for study. The parenchyma of the organ was apparently normal.

*Genito-Urinary System* The *kidneys* were involved in 6 of 8 cases. In common with the other tissues, the lesion was confined largely to the periphery of blood vessels, notably the subintimal zone of the larger veins in the columns of Bertini (fig. 25). Aggregates of lymphocytes were also found in the interstices of the cortex (fig. 24). There were no alterations in the nephrons except in the case of a transfusion reaction, where a hemoglobinuric nephrosis (lower nephron nephrosis) was present.

The 2 sections of *urinary bladder*, 3 of *prostate*, and 2 of *testis* all presented interstitial foci of lymphocytes in the neighborhood of small blood vessels.

*Endocrine System* The *adrenal* was examined in 7 cases, 5 of which displayed clusters of normal and abnormal lymphocytes beneath the intima and in the periphery of the central vein (fig. 26) as well as in the capsule. The occurrence of lymphocytes

in the adrenal is so common in the average series of autopsies that it is difficult to evaluate this finding as related to infectious mononucleosis.

The lymphoid tissue of the *thymus* was not hyperplastic in either of the 2 cases examined, and the organs were not enlarged grossly. In one of the glands, there was a perivascular reaction in which abnormal lymphocytes were present (fig. 27), the findings in the other being equivocal.

Two of the 4 *pituitary glands* displayed prominent aggregates of lymphocytes in the interstices (fig. 28), a third showing a few such cells and the fourth being essentially normal.

*Nervous System* The central nervous tissues and peripheral nerves in our Cases 1 and 2 have been described in detail by Ricker et al.,<sup>27</sup> and adequately illustrated. In summary, the meninges were congested and edematous and contained moderate numbers of mononuclear cells (fig. 29). Occasional small perivascular hemorrhages were noted within the brain substance, as well as mild ganglion cell degeneration, occasionally with satellitosis. Changes in the spinal cord were minor, but cellular infiltration of anterior nerve roots was noted at all levels, particularly in the periphery of blood vessels (fig. 32). The myelin sheaths were swollen and disrupted. In 3 other cases, distinct perivascular cuffing with round cells had occurred in the brain (figs. 30 and 31) and in 2 of these the meninges were similarly affected. An additional case displayed no demonstrable involvement. Thus, in 5 of 6 cases there was evidence of a mild to moderate meningo-encephalitis, and in 2 a distinct peripheral neuritis.

*Musculature* Sections of voluntary muscle were examined in 2 cases, one appearing normal, the other showing lymphocytic collars in the periphery of small blood vessels.

*Integument* Two biopsies of skin revealed a relatively normal epidermis save for a lymphocytic infiltration of some of the rete pegs (fig. 33). The corium was rather edematous and hyperemic, and an infiltration of small and large lymphocytes was noticeable in the vascular peripheries.

#### CLINICO-PATHOLOGIC CORRELATIONS

We have shown that there are as many lesions of infectious mononucleosis as there are organs and tissues of the body, although the degree of involvement of each varies markedly from case to case. This is manifest clinically by the wide range of signs and symptoms listed in the many series of cases reported in the literature. We have selected Read and Helwig's<sup>26</sup> analysis of 300 Army cases to illustrate this, and to show that virtually all of the clinical features may be explained on the basis of demonstrable pathologic changes. Tables 2, 3 and 4 are taken directly from their article, although the items have been rearranged by systems to conform with the scheme used for our pathologic descriptions. In the following paragraphs, we will also add certain features of the disease not mentioned in Read and Helwig's series. It is interesting to note that most of the clinical manifestations of the disease serve to confuse rather than to clarify the diagnosis.

*Hematopoietic System* In view of the fact that hyperplasia of lymphoid tissue is the major and most consistent pathologic change in infectious mononucleosis it is

TABLE 2 — *Admission Diagnoses*

Hematopoietic System	
Infectious mononucleosis	37
Lymphadenitis	10
Respiratory System	
Epistaxis	4
Acute sinusitis	12
Nasopharyngitis	45
Acute pharyngitis	64
Acute tonsillitis	36
Bronchitis	10
Atypical pneumonia	19
Influenza	4
Digestive System	
Vincent's angina	5
Gastro-enteritis	3
Jaundice	6
Nervous System	
Psychoneurosis	1
Suspected meningitis	3
Heat exhaustion	2
Miscellaneous	
Malaria	7
Reaction to typhoid vaccine	1
Cervical mass	2
Cervical myositis	5
Diagnosis uncertain	12

TABLE 3 — *Admission Complaints*

Hematopoietic System	
Lymphadenopathy	81
Respiratory System	
Epistaxis	6
Sore throat	146
Cough	38
Digestive System	
Anorexia	58
Nausea	9
Vomiting	7
Abdominal pain	14
Jaundice	6
Nervous System	
Headache	71
Vertigo	9
Miscellaneous	
Malaise	72
Fever	67
Arthralgia	7
Myalgia	5
Cutaneous eruption	11

readily understood why enlargement of the *lymph nodes* and *spleen* are the most commonly observed physical findings. The rapidity with which the enlargement develops accounts for the tenderness of these organs. We have shown in a previous article<sup>29</sup> that the tense, swollen spleen of infectious mononucleosis is peculiarly liable to rupture because the capsule and trabecular are more or less diluted and sometimes dissolved by lymphocytic infiltration.

TABLE 4 — *Positive Signs*

Hematopoietic System	
Generalized adenopathy	172
Cervical adenopathy	123
Tender adenopathy	67
Palpable spleen	104
Tender spleen	39
Petechiae	9
Oral cavity	4
Generalized	9
Respiratory System	
Epistaxis	6
Follicular pharyngitis	112
Membranous pharyngitis	34
Acute tonsillitis	29
Peritonsillar abscess	7
Hemoptysis	3
Digestive System	
Gingivitis	37
Vomiting	7
Diarrhea	4
Palpable liver	47
Jaundice	11
Abdominal tenderness	3
Nervous System	
Stiff neck	2
Mild stupor	3
Delirium	1
Miscellaneous	
Myositis	5
Dermatitis	16

Erythropoiesis in the *bone marrow* in nearly all cases appears to be essentially normal, thus explaining the rarity of anemia in infectious mononucleosis. The cause of anemia in the few cases reported was not apparent, in other instances, the anemia was regarded as coincidental. Granulocyte components are either normal or increased in number, and may show some degree of immaturity and toxic granulation (Limarzi et al.) Study of cases with actual neutropenia has been inadequate to demonstrate any significant change in the marrow. Neutrophilic leukocytosis fol-

lowing rupture of the spleen or with associated pyogenic infection may occur very rapidly and demonstrates the responsiveness of the marrow, sometimes masking the original lymphocytosis

Minot<sup>22, 23</sup> was the first to describe the striking thrombocytopenia with hemorrhagic purpura which occasionally complicates infectious mononucleosis and then regresses during convalescence. As Minot's<sup>22</sup> case was observed before the heterophil antibody reaction had been described, he hesitated to make a precise diagnosis, but in view of recent reports<sup>18 20 30</sup> there can be no doubt that this was infectious mononucleosis. One of our patients (Case 4) who died following rupture of the spleen had a condition of this type, but unfortunately bone marrow was not removed at autopsy. The only marrow study of the thrombocytopenic variant which we have found was reported by Dameshek and Grassi<sup>6</sup> whose patient recovered after splenectomy, they described an increase in megakaryocytes which showed "greatly diminished platelet production."

*Respiratory System* The essential lesion in the nose and throat is lymphoid hyperplasia which may subside uncomplicated by secondary infection or necrosis and without undue discomfort. In other instances, however, ulceration supervenes with extension of secondary invaders to produce membranous pharyngitis, peritonsillar abscesses, and even Ludwig's angina. Epistaxis probably results from ulceration and necrosis of hyperplastic lymphoid tissue in the nose, tissue was not available for study in our Case 9 in which death was due to nasopharyngeal hemorrhage, so that we were unable to demonstrate this. However, in Case 6, there was actual gangrene of the tonsil and posterior pharyngeal wall, and in Case 1, necrosis of tonsils to a lesser degree. Occasionally the lymphoid overgrowth may be so exuberant as to simulate a malignant tumor, as in one of our patients who made an uneventful recovery.

Tidy<sup>36</sup> and Wintrobe<sup>40</sup> state that laryngeal obstruction is unknown, yet the cause of death in our Case 6 was edema of the glottis. Cough is probably due most commonly to tracheobronchitis, observed in the single specimen available to us, and to actual involvement of lung tissue which was slight in 5 of our 9 autopsies, prominent in 3, and absent in 1. It is interesting that 19 of Read and Helwig's<sup>26</sup> cases were diagnosed atypical pneumonia on admission, figs 15 and 16 make clear that interstitial pneumonitis actually can occur as a feature of infectious mononucleosis and would be indistinguishable clinically and by x-ray examination from the interstitial pneumonitis of atypical pneumonia, influenza, or even rheumatic pneumonitis. Similarly, the lobular pneumonia found in Case 9 can be linked to infectious mononucleosis only by the mononuclear exudate in the alveoli. The sometimes marked acceleration in respiratory rate is probably due to pneumonitis or pneumonia usually not recognized as part of infectious mononucleosis per se.

*Cardiovascular System* No symptoms referable to the heart are listed in Read and Helwig's<sup>26</sup> article. However, arrhythmias and tachycardia are known to occur. Wintrobe<sup>40</sup> mentions one patient in whom tachycardia and cyanosis became so pronounced that acute cardiac dilatation was suspected. Myocarditis was the stated cause of death in Jersild's<sup>14</sup> case.

Electrocardiographic evidence of heart lesions has been reported. In Evans and

Graybiel's<sup>10</sup> 4 cases the T waves were lowered or inverted in all leads suggesting pericardial involvement, with gradual return to normal during convalescence. Logue and Hansen<sup>19</sup> record first degree heart block with prolonged P-R interval, while Candel and Wheelock's<sup>4</sup> case had electrocardiographic tracings indicative of acute myocarditis.

We were unable to demonstrate pericarditis in our 9 cases, at most, there was a sparse sprinkling of lymphocytes in the subepicardial connective tissue no greater than that one frequently encounters in a series of routine autopsies. Lesions were found in the muscle, however, in 6 cases, doubtful in 1, and in Case 9 no heart sections were available for study. The foci were small in all but Case 3, where there were rather extensive residual areas of myocarditis at the time of accidental death thirty days after the onset of illness.

*Digestive System* It is virtually impossible to demonstrate an anatomic basis for the nausea, vomiting, and diarrhea occurring in a febrile illness. We did find lymphocytic infiltration of appreciable degree in the tunica propria of the stomach in 3 of 4 cases examined, and fairly marked hyperplasia of intestinal lymphoid tissue in 1 of 4 cases, this change being equivocal in 3. It is doubtful whether these observations are of clinical significance.

Abdominal pain and tenderness are probably due to rapid enlargement of the spleen, liver, and abdominal lymph nodes.

Physical examination of patients with infectious mononucleosis frequently discloses an enlarged liver. In our 4 cases in which figures were recorded, the organ weighed 1650 Gm, 2100 Gm, 2600 Gm, and 2738 Gm. The normal-sized liver was from a convalescent case, whereas the patient with the largest liver died from a pulmonary embolism five days after operation for ruptured spleen on approximately the thirty-fifth day of the disease. It should be mentioned that hepatomegaly is not usually a feature of the milder case.

Liver function tests reported by Cohn and Lidman<sup>6</sup> in a series of 15 consecutive cases of proven infectious mononucleosis without jaundice gave evidence of liver damage; they found the thymol turbidity and bromsulfalein excretion tests of most consistent value. "There appeared to be a rough correlation between the severity of the disease and the degree of hepatic impairment." Their results were confirmed by DeMarsh and Alt<sup>9</sup> in 19 additional cases. Jaundice is not a rare manifestation of infectious mononucleosis and provides further evidence of liver involvement. Changes in the liver in jaundiced cases as described by Bang and Wanscher<sup>3</sup> do not differ from those in patients who have not been jaundiced. They found no reason to regard the jaundice as due to obstruction.

The liver lesion is essentially a periportal hepatitis. It is hardly distinguishable from liver changes in the milder cases of epidemic hepatitis as observed at biopsy. Necrosis of liver cells was observed in only 1 of our fatal cases, and was apparently due to portal vein thrombosis incident to splenectomy rather than to infectious mononucleosis per se. There may, however, be some slight loss of liver cells in the periphery of heavily infiltrated portal areas.

*Genito-Urinary System* Albuminuria is common, as in any febrile disease, leukocytes and red blood cells are not infrequent urinary findings, but casts are not generally seen. Gross hematuria was found in 6 per cent of Tidy and Morley's<sup>21</sup> 270

cases, apparently occurring as an isolated phenomenon, it may also appear as part of the general hemorrhagic manifestations in the thrombocytopenic variant. The renal lesion of infectious mononucleosis is essentially an interstitial nephritis. As for the genitalia, uterine bleeding in the hemorrhagic form of the disease is the only symptom of which we are aware, in Dameshek and Grassi's<sup>6</sup> case there was also a suggestion of a pre-existing hemorrhagic tendency.

*Nervous System* Although Read and Helwig<sup>26</sup> do not mention headache in their series, this is a very common symptom, often associated with blurring of vision and vertigo, and occasionally with stiff neck. Stupor, coma, delirium, and convulsions have been observed, as well as paresthesias, motor paralyses, and depression of the respiratory center. Two of our fatal cases presented the Guillain-Barré syndrome. A wide variety of neurologic signs has been reported<sup>13, 27, 28, 32</sup>. The cerebrospinal fluid findings are variable, more frequently being normal, the pressure is sometimes increased, and a lymphocytosis of several hundred cells and increase in protein have been observed. The differential diagnosis between infectious mononucleosis and lymphocytic choriomeningitis may be difficult under such circumstances. Tidy<sup>35</sup> has attempted to relate the two diseases, but Viets and Warren<sup>39</sup> state that in lymphocytic choriomeningitis, all organs except the central nervous system are relatively normal, and describe "inclusion bodies" in ganglion cells, more extensive meningeal and perivascular lymphocytic infiltrations, perivascular hemorrhage, and "glial nodules."

We have demonstrated varying degrees of encephalitis, meningitis, or both, in 3 of 4 cases in which no symptoms referable to the central nervous system were recognized. However, one of these patients lost his life in the crash of an airplane which he was piloting during the convalescent period, and the disease may have been a factor leading to the accident. In our 2 cases presenting the Guillain-Barré syndrome, there was the additional involvement of spinal nerve roots but only minor ganglion cell changes in the cord proper, peripheral nerves were examined in 1 of these cases and showed lesions similar to those of the nerve roots.

*Musculature* Myalgia is an uncommon symptom, although we found myositis in 1 of 2 cases in which skeletal muscle was examined.

*Integument* Cutaneous eruptions of various types, usually morbilliform, are frequently encountered in infectious mononucleosis.<sup>31</sup> Our 2 biopsies from such cases disclosed hyperemia, edema, and cellular reaction in the corium.

#### SUMMARY

This pathologic study is based on 9 autopsies and many biopsies in cases of infectious mononucleosis.

The gross changes were almost exclusively confined to enlargement of lymphoid tissues, especially the spleen. Nasopharyngeal lymphoid hyperplasia was constant, in one instance suggesting tumor. Other tissues presented no significant gross features related to the primary disease except for (1) rather consistent enlargement of the liver, (2) infrequent icterus, and (3) occasional cutaneous rash. Histologic observations revealed more or less generalized lesions resembling those of certain known virus diseases, notably perivascular aggregates of normal and abnormal lymphocytes. Reaction of this type inconstantly involved all tissues studied except



the bone marrow, here lymphocytes were virtually absent in sections, but were present in aspirated marrow because of dilution with peripheral blood

More specific changes were invariably noted in lymphoid tissues. The abnormal lymphocyte characteristic of the disease could be identified in thin, lightly stained sections. Lymph node reactions varied from a predominantly follicular hyperplasia to a blurred pattern simulating a malignant lymphoma, the latter was due to a lymphocytic and reticulo-endothelial proliferation in the medullary cords. The spleen displayed a lymphocytic infiltration in the thinned capsule and trabeculae, frequently dissolving the latter and rendering the organ liable to rupture. The pattern was partially effaced in most instances and the follicles widely spaced. Blood sinuses contained considerable numbers of normal and abnormal lymphocytes, and accumulations of these cells constantly cuffed intratrabecular arteries and lay beneath the intima of veins. Tonsils displayed ulceration and necrosis in several cases, and the lymphocytic proliferation closely resembled malignant tumor in a tonsil that enlarged rapidly.

A pneumonic exudate in 1 case was almost exclusively of round cell type, while in another the pneumonia was of the usual lobular type with a neutrophilic exudate. Small myocardial infiltrates which we noted probably explain the electrocardiographic changes described in infectious mononucleosis. Other findings of particular interest were the periportal lymphoid collars in the liver which sometimes attained the proportions seen in leukemia, and the presence of meningo-encephalitis in 4 of the 6 brains examined.

We believe that the majority of cells in the lymphocytic "infiltrates" of connective tissues and the perivascular collars are metaplastic rather than inwandering, i.e., that they are formed in situ and stem from cells of the reticulo-endothelial system.

#### ADDENDUM

Since this paper was prepared, our Case 3 has been reported separately by Allen and Kellner.<sup>1</sup> We have held the case in our series, however, as it lends strength to our tabulated findings.

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## CHRONIC INFECTIOUS MONONUCLEOSIS

By RAPHAEL ISAACS, M.A., M.D

IN A GROUP of 206 patients who had infectious mononucleosis, 53 had some symptoms which persisted for from three months to at least four years or longer. The syndrome included ease of fatigue, exhaustion, aching of the legs, weakness, depression, afternoon elevation of temperature (99.8 to 101 F), moderate splenomegaly, low blood pressure, low blood sugar, often low specific gravity of the urine, and the presence of infectious mononucleosis cells in the blood.

The 53 patients with the chronic symptoms included 22 males and 31 females. The ages ranged from 8 months to 60 years. There were 5 younger than 19 years, 20-29 years, 12, 30-39 years, 16, 40-49 years, 15, 50-60 years, 5. The 8 month old child showed unusual diarrhea ("dysentery") since birth, and examination of the blood showed infectious mononucleosis cells. The mother had acute infectious mononucleosis during pregnancy, one month before the birth of the child. Thirteen of the patients had the symptoms for from 3 to 6 months, 15 for 7-12 months, 3 for 17-18 months, 11 for one year, 8 from two and one-half to four years and 3 for at least six years. Some of the patients could give the exact date of the start of the symptoms, others within a month or so. Five gave the duration as "several months," "several years," "many years."

These patients had been sent in for study, with possible diagnoses of undulant fever, tuberculosis, Addison's disease, Hodgkin's disease, Rocky Mountain spotted fever, lymphosarcoma, hypothyroidism, menopausal syndrome, subacute bacterial endocarditis, neurasthenia and syphilis.

All of the group showed infectious mononucleosis cells in the blood. The red blood cell and leukocyte counts and hemoglobin content were within normal limits. The infectious mononucleosis cells were of the mature type, with deeply basophilic cytoplasm, staining the peculiar blue characteristic of these cells. The nuclei showed the streaky chromatin, with fenestrations, and were often indented. Occasionally one or more of the large forms found in the acute type were noted. The cells constituted 1 to 7 per cent of the total leukocytes, rarely higher. These cells had been grouped with the lymphocytes or monocytes by uncritical technicians.

In no case of the chronic group was the sheep cell (heterophile) agglutination titer above 1:64. Five patients showed persistent "positive" Kahn tests, characterized as "general biologic reaction," for one to six years.

The presenting symptom was always weakness or ease of fatigue. The patients said that their legs were weak and ached. The fatigue was usually present on arising in the morning, but occasionally developed late in the morning or during the afternoon. Some had symptoms suggestive of hypoglycemia. Others had mental depression, nervousness, ease of perspiration and dizzy or giddy spells on arising.

The fatigue appeared out of proportion to the physical data. The usual findings

were a slightly enlarged spleen about 14 to 16 centimeters, occasionally palpable on deep inspiration depending on the patient's body-build. The spleen could be outlined by direct percussion (not by the intermediate method) by using very light tapping. It could be demonstrated by x-ray examination. The enlarged spleen elevated the cardiac apex, while the patient was lying down, so that the cardiac tip was from 10 to 11 cm. to the left of the midline. On standing the apex lowered to about 9 cm. to the left of the midline.

The second feature noted in most of the patients was the comparatively low blood pressure. The systolic figures were from 95 to 105, occasionally as high as 115, with diastolic pressures of 56 to 70. This was significant, as most of the patients belonged to the older age group. There were no definite signs of myocardial or circulatory insufficiency, and there was usually no edema of the ankles.

In most of the patients who made observations on their temperature, there was an afternoon rise to 99.6-101.4 F. Rarely was it higher than this, but in some the elevation was so persistent that they had been diagnosed 'fever of unknown origin' or were suspected of having undulant fever, Hodgkin's disease or tuberculosis.

Most of the patients had some lymph nodes which were palpable, but never very large. Enlarged posterior cervical nodes were the most common. In many, the nodes were well within normal limits of size.

A number of patients had symptoms suggestive of hypoglycemia. Late in the morning there was a feeling of exhaustion, anxiety, increased perspiration. The blood sugar (fasting, after food, and at intervals after sugar ingestion) was observed in ten individuals. The fasting blood sugar showed levels of 33 to 70 mg per 100 cc. using a method in which most normal individuals showed from 80 to 110 mg per 100 cc. Isolated observations on other patients in the group showed levels of 80 to 95 mg per 100 cc. After ingestion of a meal or a measured amount of glucose, there was but slight increase in the height of the glucose curve (increased tolerance), and the fall was slow, although 3 individuals showed a lower level than the fasting level between three and five hours after the ingestion of the glucose.

The serum sodium, potassium and chlorine was within normal limits in these patients. None of the patients showed unusual pigmentation.

In 12 of the patients, low basal metabolic percentage had led their physicians to prescribe thyroid, without therapeutic advantage, however, as the abnormal fatigue persisted.

The only feature of the urine which was present in most of the individuals was a low specific gravity of individual specimens taken at random during the morning or afternoon. Values from 1.001 to 1.008 were common, and values higher than 1.010 were unusual in this group.

In 3 patients of the group being studied, although the onset was typical of acute infectious mononucleosis, material from lymph nodes had been obtained by biopsy. The sections were variously interpreted by different observers, and x-ray therapy was given over all the glandular areas by the patients' doctors. These patients later

had a recurrence of the glandular enlargements, and one was diagnosed as lymphosarcoma, one reticulum cell sarcoma and one Hodgkin's disease. They continued to show clear-cut infectious mononucleosis cells in their blood. The "lymphosarcoma" patient received intensive x-ray irradiation from several doctors, as well as "nitrogen mustard" until he died of emaciation. The results make one wonder if lymph nodes, injured or made more susceptible by infectious mononucleosis, may be made to show "malignant" characteristics after x-ray therapy.

In another group, 2 patients showed progressive enlargement of the spleen and were later classed as "Banti's disease." It is possible that some congestive splenomegalies may arise in this way.

In the differential diagnosis, undulant fever presents the most difficult problem. The various tests for degrees of immunity are not diagnostic of the disease, and a positive blood culture is not easily obtainable in the chronic form. The presence of infectious mononucleosis cells in the blood is a differential point, although an individual could have had both diseases. When the Kahn test was positive in these patients, it was of the "general biologic" type.

#### TREATMENT

Many therapeutic agents were tested without success. These included caffeine, amphetamine sulfate (benzedrine and dexedrine), strychnine, thiamine, atropine, multiple vitamin mixtures, thyroid, ephedrine and special diets. These substances produced no lasting effect, and often accentuated the nervousness. The most promising medicine appeared to be a preparation of adrenal cortical extract (cortalex). This was given in doses of 2 tablets (made from aqueous extract of 10 grams of adrenal gland) on arising in the morning. There was but little subjective improvement during the first week, but a definite feeling of well being developed during the second week and was quite definite during the third week. After this the medication was discontinued and the improvement usually continued. In a few patients it was necessary to increase the dose, or resume it after its discontinuance. Associated with the subjective improvement, there was a decrease in the size of the spleen. The changes in the blood pressure were slight.

The fact that the symptomatology is somewhat suggestive of adrenal insufficiency of the Addison's disease type, and that administration of adrenal cortical extract by mouth relieved the patients after the symptoms had persisted for long periods, suggests a possible mechanism for the fatigue during the chronic stage. There are apparently no data on the appearance of the adrenal cortex in this condition, and whatever damage is present must be reversible, if adrenal insufficiency is the cause of the symptomatology.

#### SUMMARY AND CONCLUSIONS

A group of patients is described in whom ease of fatigue, fever, splenomegaly, low blood pressure, low blood sugar, low specific gravity of the urine and the presence of infectious mononucleosis cells in the blood persisted for from three months to longer than four years after the initial attack.

Three of the group developed characteristics of lymphoblastoma and two showed the features of Banti's congestive splenomegaly.

The symptoms responded to treatment with a preparation of adrenal cortical extract.

The syndrome is apparently not uncommon and the intense, prolonged debility, together with the marked improvement after therapy with adrenal cortical extract, makes its recognition of great practical importance.

# IS IT POSSIBLE TO TRANSMIT OR ACCELERATE THE DEVELOPMENT OF MOUSE LEUKEMIA BY TISSUE EXTRACTS?

By J ENGELBRETH-HOLM, M.D.

**D**URING the last ten years it has been reported from various laboratories that the injection of certain tissue extracts has been followed by accelerated development of spontaneous leukemia in mice, the disease presenting an increased incidence and (or) an earlier appearance in the experimental animals than in the untreated controls. Although these observations lack satisfactory confirmation, it seems desirable to review them here, and to record some supplementary investigations bearing on the same problem.

In the Year Book of the Carnegie Institute, New York, for 1937, MacDowell and collaborators described experiments in which monthly injections of embryonic tissue extract into mice of the strain C 58 (with a 90 per cent leukemia incidence) were followed by development of the disease in all of the 60 experimental animals at an earlier date than in the controls belonging to the same litters. The results were not reported in detail, nor was it stated whether the test has been repeated. Gorer, who tried to confirm this finding, states merely "Inoculations of embryonic tissue have had no noticeable effect on either the 'albino' or the 'black' leukemia."

In 1938, Engelbreth-Holm and Frederiksen believed they had transmitted mouse leukemia to young animals of the strain Aka by means of a cell-free extract of leukemic organs from mice of the same strain. The extract was prepared under anaerobic conditions reduced in a cysteine-cobalt-sulphate system as described by Pirie and Holmes. Injection of the extract was followed by the development of leukemia in 8 experiments out of 9, totalling 36 mice out of 179. The tests were carefully controlled in various ways. Thus, *aerobically* prepared extracts showed no effect in 5 experiments including 120 animals. Further, a minimum of 1000 cells was found to be necessary to secure a "take" in the ordinary way, it therefore seemed impossible that the "takes" in these experiments could have been due to presence of sufficient intact cells in the extract, since the latter had been centrifuged twice for fifteen minutes at 3,000 r p m. As a most deplorable fault, it must be noted that the extract was not filtered in these tests. Engelbreth-Holm later (in 1942) expressed the view that the findings in these experiments might have been a question of acceleration of spontaneous leukemia rather than of a transmission of the disease.

MacDowell and his collaborators (1939) tried to repeat the observations of Engelbreth-Holm and Frederiksen without success. Still more confusion however, was brought into the matter when, after control injection of the medium used for reduction (a cobalt-sulphate-cysteine solution), MacDowell found leukemia developing in 17 out of 20 mice only twenty-six to thirty-eight days later. Repetition of this experiment gave negative results.

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Finally, Gorer in 1939 reported a somewhat analogous observation. Inoculation into mice of a "nontaking" sarcomatous tissue appeared to increase the leukemia incidence from 6 to 39 per cent in all animals, and from 2 to 46 per cent in the males. Re-inoculation brought about a still greater rise in incidence. Gorer inoculated a sarcoma from an albino strain into a black mouse strain. After repeated inoculations, leukemia developed in 7 out of 10 mice, whereas the spontaneous leukemia incidence was only 6 per cent. Gorer found that the leukemia did not develop until about one year after the inoculations, i. e., probably at the same age at which the disease will appear in untreated mice.

In an attempt to explain the results of our original experiments (Engelbreth-Holm and Frederiksen), new investigations along the same lines were performed during the years 1938-42. Our efforts, however, were no more successful than those of MacDowell. We did not observe any acceleration of the leukemia in mice belonging to the strain Aka after injection of an extract of leukemic organs prepared under anaerobic conditions. Repeated injections of such extracts were made in 25 mice belong to the strain Aka, a total of 17 injections being administered at intervals of two weeks. For control purpose, extracts of normal organs were injected into brothers and sisters of the experimental animals, but no effect was seen in either of the two series.

Likewise, MacDowell's experiments with the cobalt-sulphate-cysteine solution were repeated. Forty-eight Aka-mice were given 1 cc. of the solution, the mice at the beginning of the experiment were one month old, and the injections were administered every two weeks until they died spontaneously. In 23 out of these 48 mice, leukemia developed, but among their 44 untreated brothers and sisters, 23 cases of leukemia were found as well, the treatment, therefore, had had no evident effect.

No more did we succeed in accelerating other tumor types: leukemia and mammary carcinoma of the strain dlb. These experiments, comprising 38 and 15 mice respectively, were equally negative.

The most natural way in which to explain these rather capricious and mostly negative results was to assume that, despite the precautions taken, the spun extract in our 1938 experiments did contain a number of intact cells sufficient to secure "takes", this explanation still failed to account, however, for the fact that administration of *aerobically* prepared extracts did not give any "takes". In spite of numerous unsuccessful attempts to repeat our 1938 observations, and in spite of the controls having indicated that a few intact cells in the injected substance could not explain the development of leukemia, I initiated one more experiment in order to exclude the possibility that cells when suspended in the cobalt-sulphate-cysteine solution were more capable of "taking" than when suspended in a sodium-chloride solution as in the original control experiments.

Known numbers of leukemic cells, suspended partly in normal saline and partly in a cobalt-cysteine solution, were accordingly injected into series of mice. The result may be seen in table 1.

No explanation was achieved by these experiments, as suspension in the reduction solution had a definite effect in suppressing the "taking" capability of the



cells (a finding which was to be expected beforehand), leaving it, thus, still more improbable that our "takes" or "accelerated cases" in the experiments of 1938 could have been due to the presence of intact cells in the extract solution

In 1946, however, a paper was published by Silber in Russia, describing investigations which may possibly throw some light on these obscure questions, though it is necessary to await confirmation of the findings before reaching a final opinion. According to Silber, sarcomas induced in mice by 1,2,5,6-dibenzanthracene are transmissible by means of Berkefeld-filtered extracts of tumor tissue which has been treated according to the method introduced by Engelbreth-Holm and Frederiksen (dissection in a closed chamber filled with carbon dioxide, and suspension in a cysteine-cobalt system in order to prevent oxidation)

In 4 out of 5 experiments, Silber had "takes" in a total of 18 out of 114 animals. He makes it clear, however, that the takes occurred only when the substance had been prepared from very young tumors ("incipient" sarcomas), and, further, that the experimental animals required to be "sensitized" by "subcutaneous injection of 0.5 cc. of an oily solution of Dibenzanthracene containing 1 mg. of this substance in one liter of vegetable oil. This injection was administered 1-2 weeks before the test." Without this "sensitization" no takes were seen.

TABLE 1

Number of leukemic cells	Suspended in saline "Takes"/number of mice	Suspended in cobalt-cysteine solution "Takes"/number of mice
1,000,000	1/2	3/5
170,000	5/5	0/5
30,000	2/5	0/5
5,000	0/5	0/5

An observation by Duran-Reynals in fowl sarcomas may also prove of interest in this discussion. Duran-Reynals has pointed out that the virus of fibrosarcomas are detected more frequently at the age of 5 to 10 months than in younger or older fowls.

Whether or not these findings have any bearing on "transmission" of leukemia in mice cannot yet be decided. In our experiments, no attention was paid to the age of the donor animal or to that of the tumor tissue, nor was it possible to pay regard to such changes of character as might have taken place in the inbred mouse strains during the passages.

Attempts have further been made to repeat Gorer's experiments mentioned above. In our experiments, different tumors were inoculated into mice belonging to three different strains in which the tumors used did not take. Inoculation was made subcutaneously and repeated every two weeks (see table 2), one half of each litter being left untreated as controls. The three strains employed were the strain Aka, the strain dlb, and the strain Street. The strain Aka has a spontaneous leukemia incidence of 57 per cent, in the strain dlb (subline of the Little DbA), leukemia will develop in 1 per cent, and mammary carcinoma in about 40 per cent, and in the strain Street, leukemia incidence is 1 per cent, the incidence of mammary carcinoma

being 25 per cent. The transplanted tissues in question were a mammary carcinoma from dlb-mice, a leukemic tissue from Aka-mice, and a squamous cell carcinoma from the strain Aka which had been transferred through several passages (see Engelbreth-Holm 1944)

Further details of the experiment may be seen in table 2. The treatment did not increase or accelerate tumor development, and the results of Gorer's investigation were therefore not confirmed. Unfortunately, however, owing to a fulminating epidemic, all the animals died when 17-19 months old, and it is impossible to decide how many tumors might have developed if the mice had not succumbed prematurely. Nevertheless, since tumors occurring in these strains will generally develop spontaneously from the age of 10 to 12 months, the climax being at about 15 months, it was clear that the treatment did not accelerate tumor development, both the experimental and control animals presenting only a few tumors.

TABLE 2

Tumor tissue from	Inoculation made into	Number of inoculations	Age of mice at the end of experiment
Mammary carcinoma dlb	24 Aka-mice	3	17-19
	53 Street (24 ♂ 29 ♀)	5	
Leukemic tissue Aka	55 dlb	6	18
	(34 ♂ 21 ♀)		
	52 Street (30 ♂ 22 ♀)	6	18-19
Squamous cell carcinoma Aka	49 dlb	6	17-18
	(28 ♂ 21 ♀)		
	54 Street (30 ♂ 24 ♀)	6	17

The result in each instance was No effect upon tumor development

We have thus been unable, with the strains used in these experiments, to repeat Gorer's finding that inoculation of heterologous tumor tissue can bring about an increased leukemia incidence.

#### DISCUSSION

A series of experiments is reviewed, although largely supporting each other, they have proved inaccessible to direct reproduction. The various positive investigations originally indicated that development of spontaneous leukemia in inbred mouse strains is accelerated after the injection of embryonic extract or leukemic tissue extract, or after inoculation of heterologous tumor tissue.

MacDowell and his collaborators succeeded in accelerating the leukemia incidence after administration of embryonic tissue extract. Gorer did not succeed in reproducing these experiments, but he gives few details of his negative result to which, indeed, he seems to ascribe but a limited importance.

Englebreth-Holm and Frederiksen thought that they had accelerated the development of leukemia, or transmitted the disease, by means of an anaerobically prepared extract of leukemic tissue. These findings, however, have remained refractory to reproduction in spite of repeated attempts made by MacDowell and ourselves.

MacDowell observed acceleration of leukemia in one experiment by means of a cysteine-containing suspension, but neither he nor we have been able to repeat this observation.

After transplantation of a nontaking tumor tissue, Gorer achieved an accelerated development of leukemia. Despite several attempts to repeat these experiments, however, we have not succeeded in confirming Gorer's results.

How these discrepant findings are to be explained is still obscure. The various control series have been adequate, and no experimental faults which might have produced the positive or negative results have been detected. Most peculiar is the fact that the three positive series of experiments quoted, although mutually different, show one common feature, viz. the injection of rapidly growing tissue or tissue extracts, that is, of homologous or heterologous tumor tissue, and of embryonic tissue extract.

It is a most fascinating thought that in these experiments we may be approaching a factor capable of accelerating tumor development, but undeniably, there must still be a number of factors escaping our control.

Transient changes of disease conditions in the mouse strains used can probably be excluded, to judge from the control series. Certain more recent experiments, however, may possibly throw light on these questions. Silber claims to have transmitted sarcomas in mice by means of cell-free filtrates, using the same technic as we did in our earliest experiments, but he points out that only filtrates from quite young tumors have any effect, and Duran-Reynals has shown that detection of virus in fowl sarcomas most easily will be successful in certain age groups, younger and older animals offering more difficulties.

It is premature to attempt to assess the importance of these experimental results to mouse leukemia, but they seem to call for a re-examination of the relevant problems on a wider basis.

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# IS LEUKEMIA A DISEASE OF THE RETICULO-ENDOTHELIAL SYSTEM?

By BRUCE K. WISMAN, M.D.

AT THE twenty-second year milestone following discovery of a rewarding treatment of pernicious anemia by Minot and Murphy, there is little doubt that the problem presented by leukemia is more important than any other in the field of hematology. Whether the increasing incidence in this disease is actual or only apparent due to more refinements in diagnosis (especially the widespread use of bone marrow biopsy) and more physicians interested in hematology is debatable. In any case, the frequency with which this diagnosis is made is undoubtedly rising.<sup>1</sup> This fact is highlighted by the observation that little or no real progress has been made in pathogenesis or treatment, since with unimportant exceptions, the efficiency of treatment by blood transfusions and x-ray therapy is still supreme (and unsatisfactory) and the prognosis for longevity remains unchanged. These facts suggest that a fresh, if not new, point of view with respect to this disease would not be undesirable. The present observations in a series of clinical and hematologic studies of monocytic leukemia may suggest an important role of the reticulo-endothelial system in the mechanism of the production of leukemia.

## THE RETICULO-ENDOTHELIAL SYSTEM

Although ameboid cells in the connective tissues, distinct from the blood cells, were described as long ago as at least 1863,<sup>2</sup> it remained for Aschoff in 1913, working with vital staining methods using lithium carmine, to recognize that the phagocytic cells described by various cytologists under varying names were widespread throughout the body, forming a system of cells.<sup>3-4</sup> Almost at the same time, hematologists were struggling with the problem of the identity of the monocyte, thought to be a white blood cell with exceedingly well developed powers of phagocytosis but resembling the neutrophilic leukocyte on the one hand and the lymphocyte on the other. These conflicting observations were resolved by Schilling-Torgan<sup>5</sup> by establishing that the monocyte is a separate cell type. This was the general situation until 1925 when abundant evidence began to accumulate from the study of inflamed tissue and especially from tissue culture techniques<sup>6-10</sup> that monocytes and clasmotocytes were capable of transformation from one to the other (table 1). Recently, in our laboratory, this transformation has been convincingly demonstrated by Houghton<sup>11</sup> with a single cell tissue culture technic. This observation has been further strengthened by the identification of transitional types of mononuclear phagocytic cells in human blood which, when strained supravitaly, have characteristics of both monocytes and clasmotocytes (see also fig. 6).

At present, therefore, it seems to some of us rather convincingly demonstrated that the monocyte is a derivative of the reticulo-endothelial system and that this system, having a blood as well as a tissue component, greatly exceeds in extent and

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importance that which was originally indicated by the concept of Aschoff and Kiyono Monocytic leukemia, therefore, may be regarded as fundamentally and in fact as a leukemic reticulo-endotheliosis Monocytic leukemia might, therefore, furnish a valuable approach to the study of the various reactions and potentials of the reticulo-endothelial system of cells when under intense stimulation

REACTIONS OF THE RETICULO-ENDOTHELIAL SYSTEM TERMINATING  
IN MONOCYTIC LEUKEMIA

During the past seventeen years in our clinic at Columbus, 192 cases of monocytic leukemia have been studied Many of these have shown the most unusual cytologic reactions in our entire experience with the blood dyscrasias of all types This has recently been the subject of comment <sup>12</sup> <sup>13</sup> Cases initially appearing to be

TABLE 1 —*Historical Development of Identity and Relationships of Monocyte  
Clasmatocyte and Fibroblast*

Changing concepts of the separate identity of the monocyte are shown in the left panel, of the clasmatocyte in the right panel The bottom horizontal strip indicates that, under proper environmental conditions, monocyte, clasmatocyte and fibroblast may revert from the one to the other

Monocyte	Clasmatocyte
Blood	Connective Tissue
Transitional Neutrophile (Ehrlich-Naegeli)	Clasmatocyte (Ranvier 1891)
Neutrophile does not contain azur granules (Michaelis & Wolfe 1902)	Macrophage separated from microphage (Metchnikoff 1892)
Independence of Monocyte & Neutrophile (Pappenheim & Ferrata 1911)	Adventitial Cells (Marchant 1890)
Monocyte a separate cell type (Schilling-Torgau)	Polyblast (Maximow 1902)
Monocytic leukemia described (Schilling-Torgau & Reschad 1913)	Clasmat, Macrophage, Adventitial cell and Polyblast identical (Goldman 1909)
	Histiocyte & R. E. System (Aschoff & Kiyono 1913)

Monocyte  $\rightleftharpoons$  Clasmatocyte  $\rightleftharpoons$  Fibroblast  
Tissue Culture, Lewis, Houghton etc 1925-46

myeloid, or lymphatic leukemia but terminating as classic monocytic leukemia have been seen in addition to those which showed approximately equal numbers of all three cell types throughout the entire course of the disease Instances in which there was an early stimulation of megaloblasts have been observed Polycythemia vera coexisted in one case In several instances, long remissions<sup>14</sup> with near normal hematologic recovery occurred at a time when with marked anemia and thrombocytopenia existing the disease was thought to be far advanced Examples of a few of these unusual hematologic reactions will be given in brief summarization

CASE REPORTS

Case 1 (Fig 1) This patient, a colored male, age 28, was first seen on December 30, 1946, presenting marked generalized adenopathy and splenomegaly The lymph nodes were exceedingly hard upon

pulpation and the spleen quite nodular but also very hard. Repeated bone marrow aspirations from the sternum failed to show many free cells, the bone marrow content being chiefly reticulum cells and a few monoblasts. Bits of solid bone marrow tissue, however, consisted almost entirely of reticulum cells. An occasional megakaryocyte was seen and a fair sprinkling of myeloid cells in all stages of maturation. Lymph node biopsy showed complete loss of architecture, the cellular content being composed almost

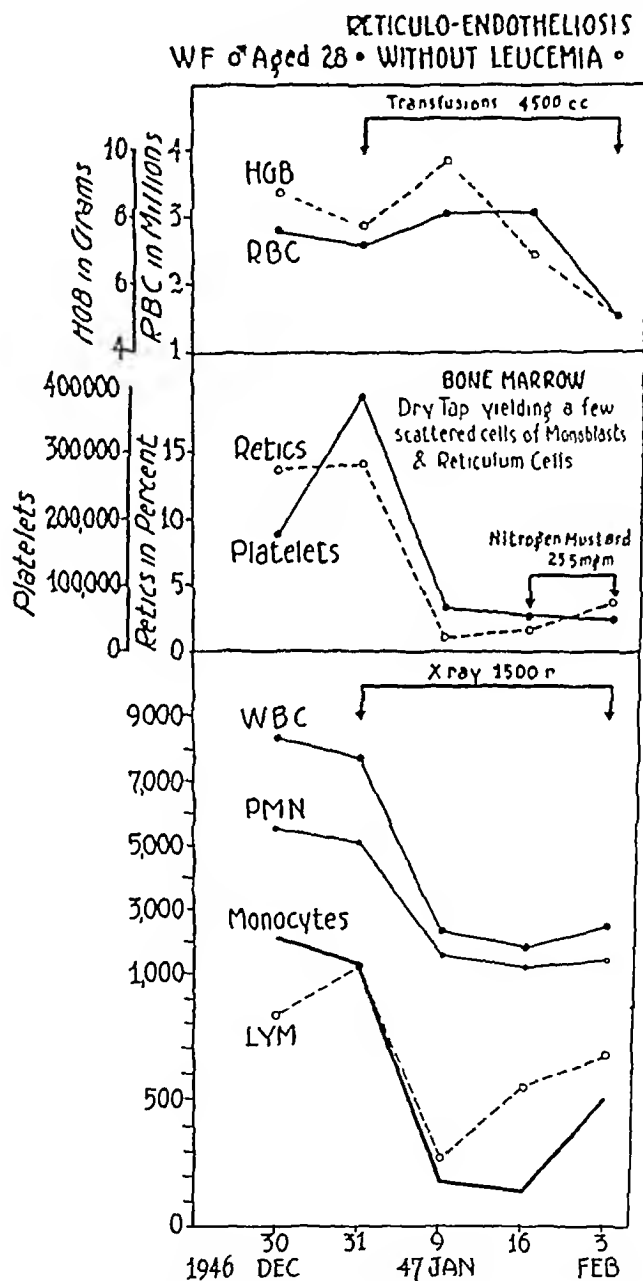


Fig 1 Graph of the hematologic reactions in a patient with "pure" reticulo-endotheliosis Case 1 of text. White cells in semi-logarithmic scale

entirely of reticulum cells and monoblasts. Reference to figure 2 shows increasing difficulty in the supply of circulating blood elements, even before the application of the nitrogen mustard and deep x-ray therapy, which, incidentally, resulted in no visible decrease in the size of the adenopathy or splenomegaly.

This case is shown as an instance of reticulo-endothelial hyperplasia with very little tendency to do other than reduplicate its own type of cell. This, therefore,

would be an instance of almost "pure" reticulo-endotheliosis, and is to be contrasted especially with the following case

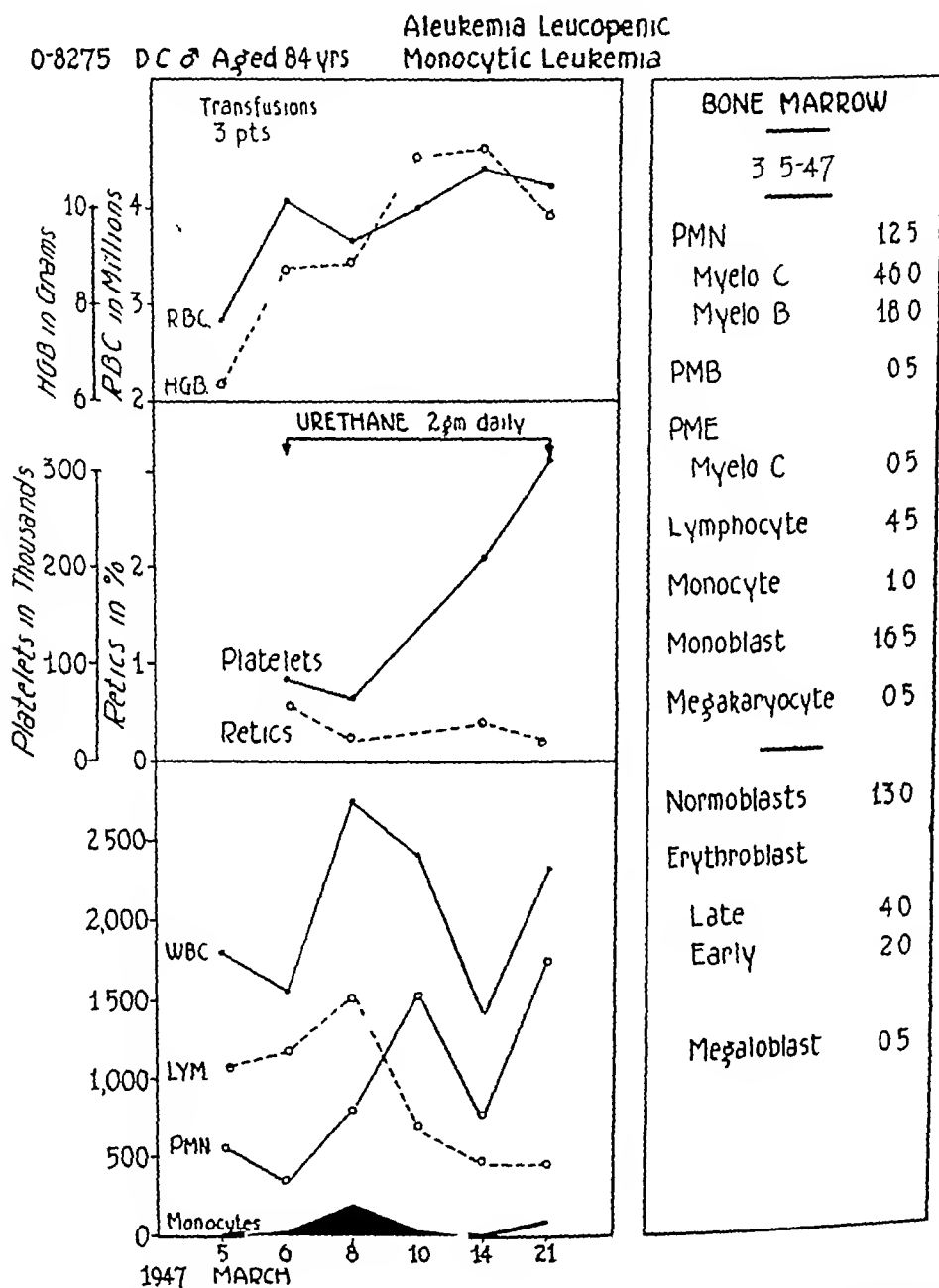


FIG 2 Graphic representation of the blood findings in a patient with aleukemic reticulo-endotheliosis Case 2 of text. The differential cell-count of the bone marrow as obtained by the aspiration technique is shown in the vertical panel on the right. Nucleated red cells listed refer to the number encountered in counting 100 white blood cells.

Case 2 (Fig 2) This patient, a white male aged 84 years, has been ill for sixteen months, during which time his clinical and hematologic state has varied little. He has received blood transfusions about once a month to maintain his red cell count. There is no apparent physical deterioration and he goes about in the same fashion as almost any man of the stated age. Figure 2 shows the hematologic record

for a typical month (March, 1947), during which time he received urethane. Although this medication apparently improved the levels of blood platelets and neutrophils, it was discontinued because the patient definitely felt worse when he was on this form of treatment. Of importance to the present discussion is the fact, clearly shown on this graph, that the level of monocytes in the blood are very low with no immature forms present at all, while the bone marrow constantly shows a small, but definite, percentage of monoblasts which does not vary appreciably from time to time.

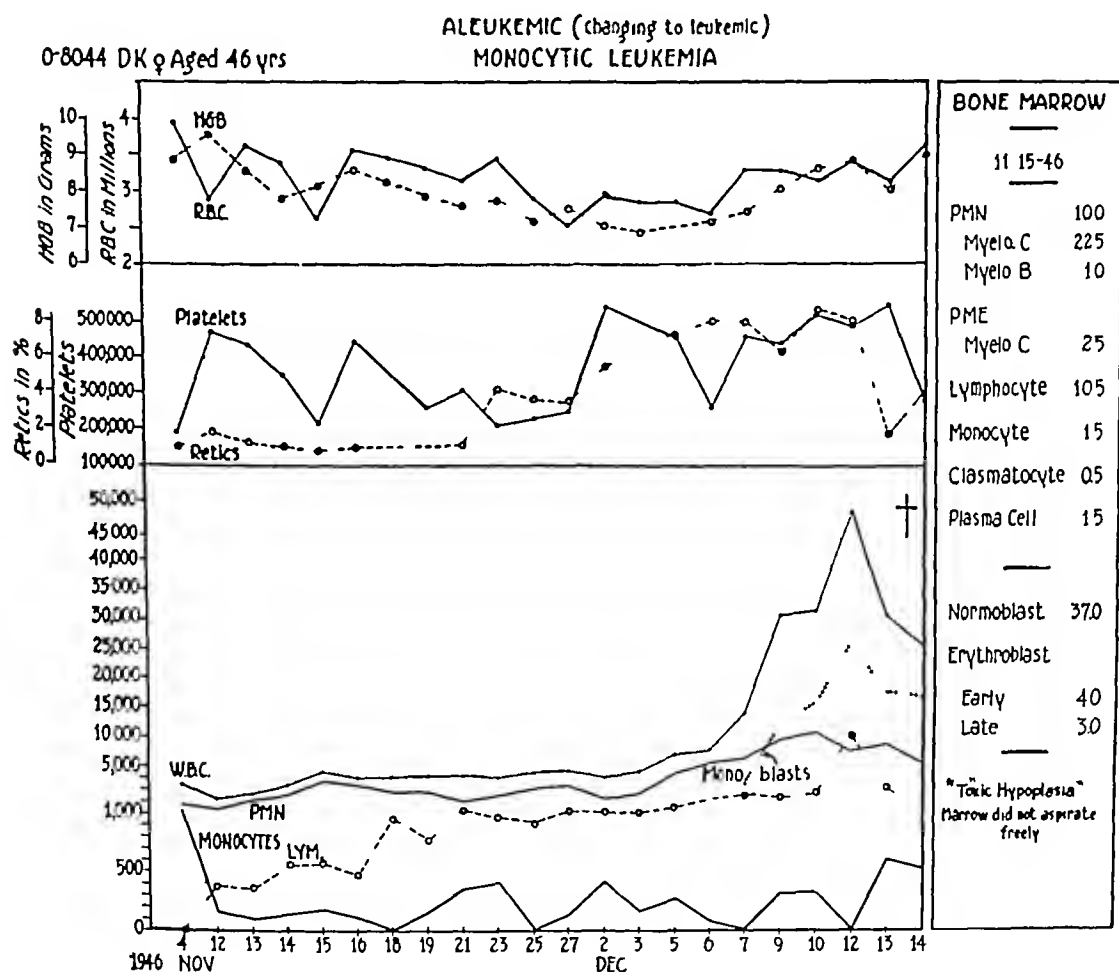


FIG 3 Graphic representation of the blood findings in a patient in which transition from aleukemic to leukemic monoblastic leukemia occurred. White cells in semi-logarithmic scale. Case 3 of text. The differential cell-count of the bone marrow in the aleukemic phase is shown in the vertical panel on the right. Nucleated red cells listed refer to the number seen in counting 50 white blood cells.

This case illustrates a minimal reaction of the reticulo-endothelial system in the direction of producing monoblasts with little tendency for maturation to monocytes. This is reflected in a clinical course that is unchanging, paralleling the stationary character of the hematologic reaction.

*Case 3 (Fig 3)* This patient, a white female, aged 46 years, illustrates the transition from an aleukemic state, in which no qualitative or quantitative changes in the blood monocytes could be detected, to a frank monoblastic leukemia. Also illustrated is the deceptiveness of attempting to interpret the bone marrow findings as obtained by the aspiration technic when the marrow does not aspirate freely. Re-



peated samplings of the marrow were attempted from the sternum but only a few drops of acellular fluid were obtained on each occasion. In this material no free monoblasts and no increase in monocytes were obtained, as shown in figure 3 (right panel). Monoblasts first appeared in the blood seven days before death, but monocytes were never increased.

**Case 4** (Fig. 4) Demonstrated here is a case of monoblastic leukemia in which, during the early phases of the disease, an appreciable number of megaloblasts were found in the bone marrow, when monoblasts were not apparent in this tissue. When leukemia first became clearly evident, small numbers of monoblasts began to appear in the marrow but megaloblasts were no longer to be found. Later, large numbers of monoblasts were present in both blood and bone marrow.

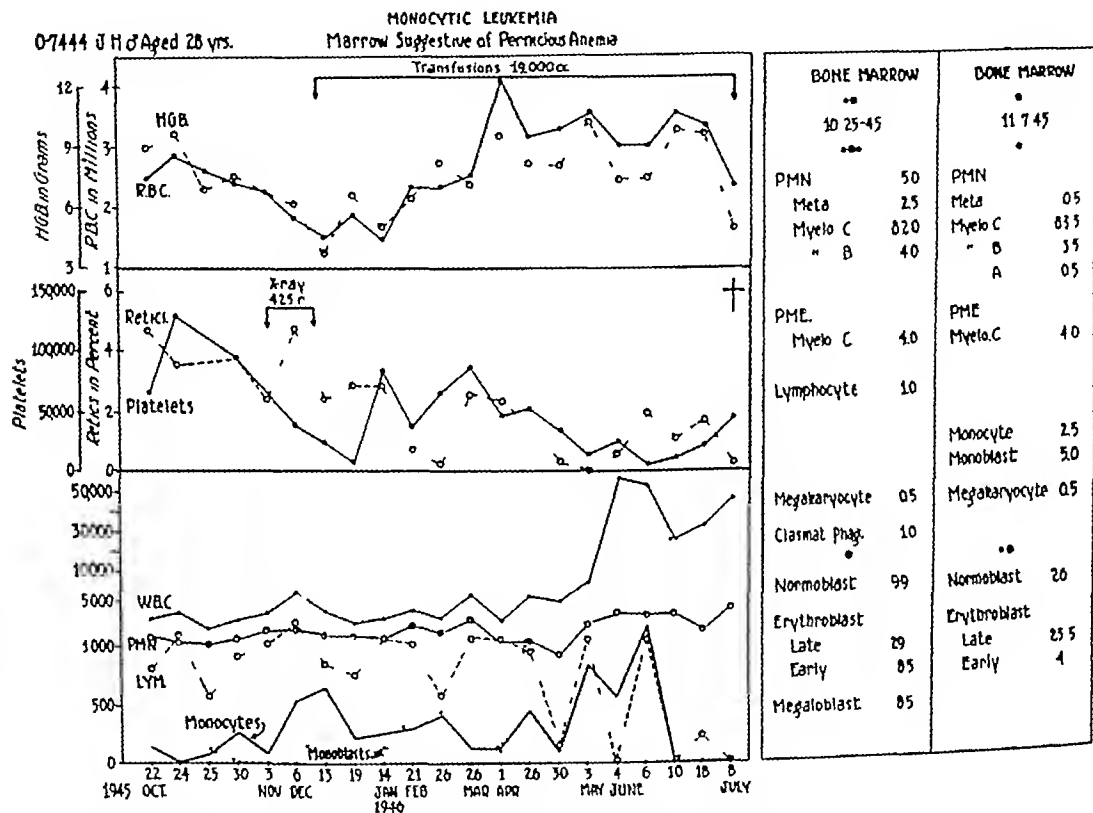


FIG. 4 Hematologic graph of a patient with monoblastic leukemia in which megaloblasts and increased numbers of early erythroblasts occurred in the bone marrow during the early course of the disease. Case 4 of text. Typical bone marrow findings are given during the early phases (first vertical panel on right of graph) and late phases (second vertical panel) of the leukemia. Nucleated red cells shown indicate number seen in counting 100 white blood cells. White cells plotted in semi-logarithmic scale.

The data in this case suggest a low grade stimulation of the reticulo-endothelial system, in the early phases of which pathologic megaloblasts were the first abnormal free cells to appear in proximity to reticulo-endothelial tissue, later, the stimulus resulted in more directional changes in terms of formation of monoblasts.

**Case 5** (Fig. 5) This case of monocytic leukemia is remarkable because of the coincident polycythemic levels of red blood cells. The patient had a large spleen (extending to the level of the umbilicus) which was removed at another hospital, the tissues being unfortunately lost. When first seen in this clinic the usual clinical signs of polycythemia vera were present, i.e., cherry red mucous membranes, liver-like tongue, distended dark retinal veins, chronic conjunctivitis, etc. There was no adenopathy.

The initial blood examination (fig. 5) showed high levels for all the circulating blood elements including reticulocytes. However, the only pathologic white cells found were monoblasts although young and mature monocytes were distinctly plentiful. Radiation therapy with radioactive phosphorus as shown

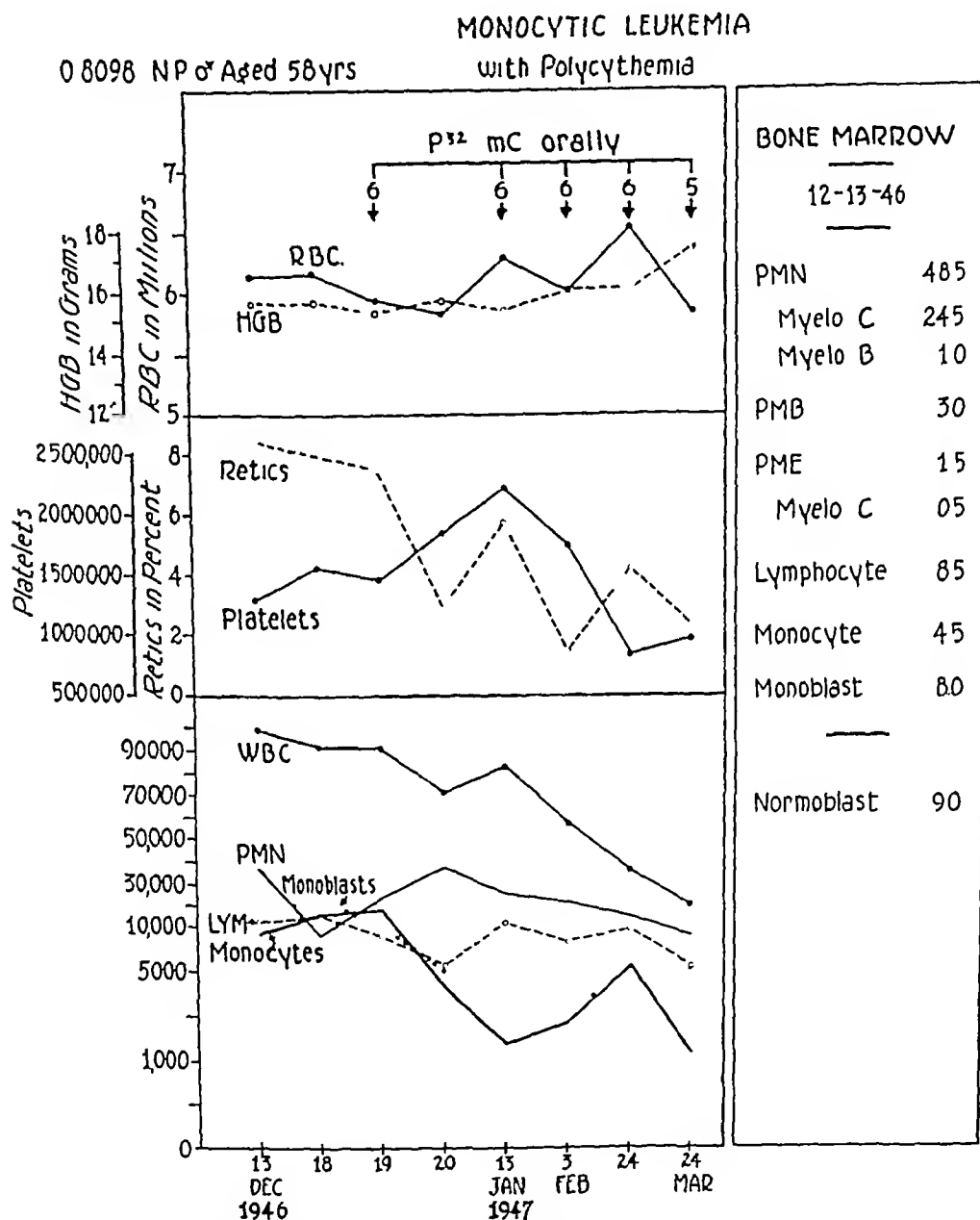


Fig. 5 Hematologic graph in a patient with monoblastic reaction occurring coincidentally with the blood and clinical findings of polycythemia vera. Case 5 of text. Bone marrow count is shown in the vertical panel on the right. Nucleated red cell elements are given as the number encountered in counting 100 white blood cells. White cells in semi-logarithmic scale.

presumably has decreased the monoblasts in the blood almost to the vanishing point so that now the patient shows little else than the classic hematologic and clinical signs of polycythemia vera.

We interpret these bizarre hematologic events to be the result of increased stimulatory effects upon the reticulo-endothelial system with dominate effect upon the

intersinusoidal reticulo-endothelial system capillaries (red cell-forming precursory tissues) of the marrow, producing increased numbers of mature erythrocytes. Elsewhere, this stimulus results in a monocytic response resembling the average

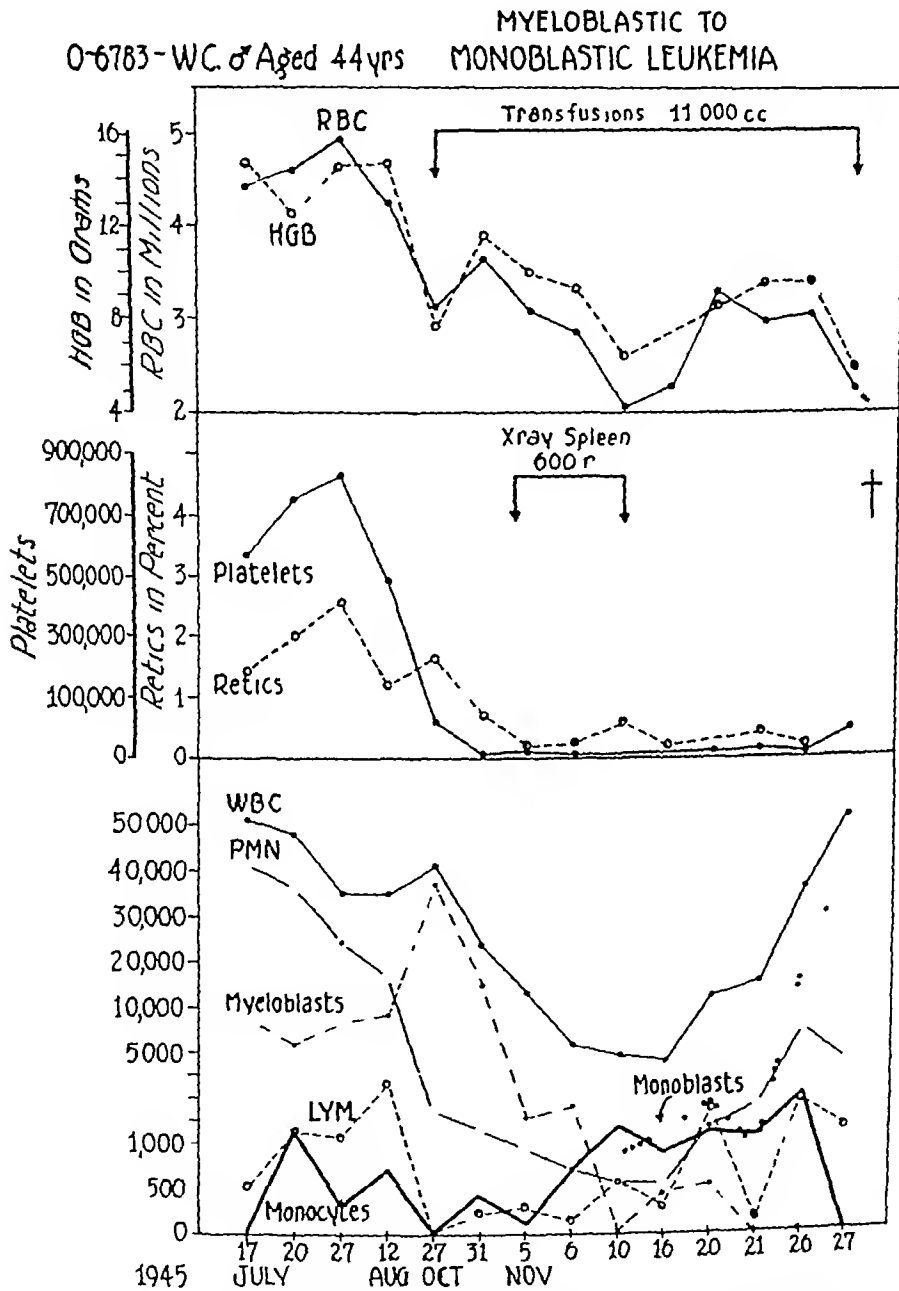


FIG. 6 Graphic representation of the blood findings in a case of myeloblastic leukemia, later terminating as monoblastic leukemia. Case 6 of text. White cells in semi-logarithmic scale.

case of monocytic leukemia except for the unusually favorable response to radiation therapy.

*Case 6 (Fig. 6)* This patient initially presented with the peripheral blood and bone marrow findings of myeloblastic leukemia. As shown in figure 6, neutrophilic leukocytes constituted 80 per cent of the circulating level of 50,000 white blood cells, indicating that our diagnosis of the immature cells present

at that time as myeloblasts and not monoblasts was probably correct. Subsequently, and coincidentally, with radiation therapy as shown, the myeloid reaction completely disappeared to be replaced by a blood and bone marrow picture of monocytic leukemia which persisted until death. Autopsy findings in this and the preceding cases were those usually noted in monocytic leukemia described in a previous publication from this<sup>15</sup> and other laboratories.<sup>16</sup> There is some evidence here that myeloblastic leukemia may

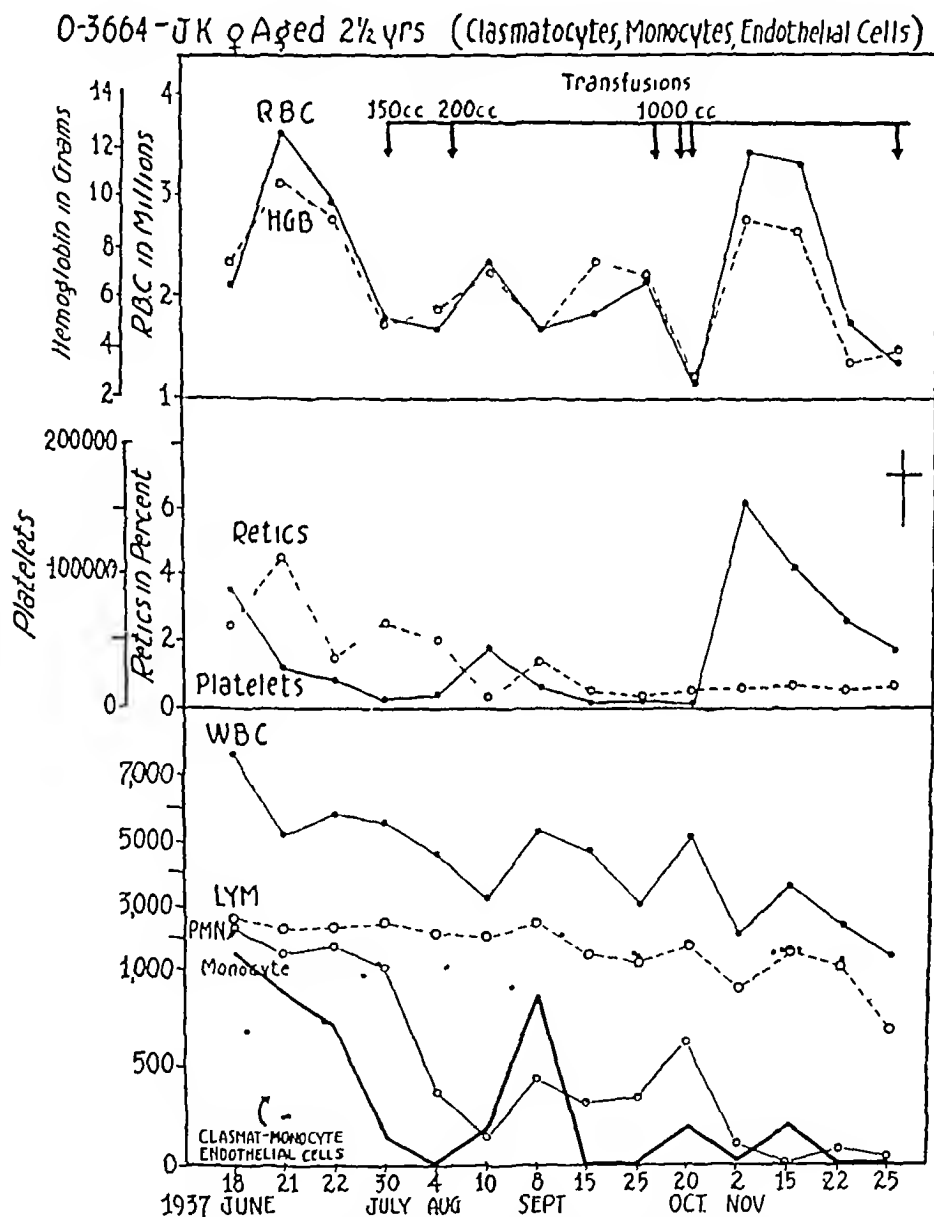


FIG 7 Clasmotocytic leukemia characterized by many transitional phagocytic mononuclear cells. Hematologic graph of a case of reticulo-endothelial leukemia. Case 7 of text. White cells in semi-logarithmic scale.

be one manifestation of reticulo-endothelial disease. If the myeloblasts did not arise originally from reticulo-endothelial stimulation, it is difficult to understand why the proliferation of these cells did not persist with the advent of the monocytic reaction.

Cases similar to the above, in which the initial cell stimulation consisted of lymphocytes with immature lymphoid elements present, and others in which im-

mature elements of all three types of white blood cells were increased in approximate equal proportions, have been previously described and reported from this laboratory<sup>15</sup> and need not receive additional emphasis in this communication

*Case 7 (Fig 7)* This patient furnishes, through the observed hematologic reactions during her illness, additional evidence that the reticulo-endothelial system may undoubtedly undergo stimulatory changes resulting in a leukemia of reticulo-endothelial cells. In this chart, only morphologically classic monocytes are labeled as such, represented by the heavy solid line. However, the dotted line of the graph represents phagocytic cells, many of which had predominating monocytic characteristics as well as cells that were definitely clasmatocytes and endothelial cells. There is little doubt that this was a leukemia of reticulo-endothelial elements in which monocytes participated as one of the pathologic cells. Blood cultures were sterile and there was no evidence of bacterial endocarditis or other sepsis.

### DISCUSSION

Doubt that monocytes are derivatives of the reticulo-endothelial system, and that monocytic leukemia is therefore not a disease of this system of cells has often been expressed,<sup>17</sup> chiefly because reticulo-endothelial hyperplasia is not always demonstrable in this disease. It should be pointed out, however, that numerical increase in the reticulum and specific endothelial cells probably will not be apparent unless maturation of these elements is obstructed. That is to say, when cell division *and maturation* occur uninhibited, hyperplasia of that cell type often is not apparent by microscopic examination of the tissue in question, the numerical increase is noted only in the end-state cell. An excellent example of a tissue reaction supporting this statement is furnished by almost universally accepted observations in pernicious anemia. During the phase of relapse, megaloblastic hyperplasia with the separate power of division of the cell intact is outstanding in the near absence of maturative principle. When the maturative principle is supplied, however, megaloblasts rapidly disappear, so that within forty eight hours and thereafter, no greater number of megaloblasts can be found in the bone marrow than is apparent in a normal resting marrow. Within seven days, however, mature red blood cells are being supplied to the circulation in maximum numbers, i.e., only the end cell product is visibly increased, although little doubt can be entertained that these new red cells are taking origin primarily from the megaloblasts. There is little reason therefore to demand visible evidence of reticulo-endothelial hyperplasia in monocytic leukemia to satisfy the hypothesis that in this disease the monocytes take origin from the reticulo-endothelial system. *In a blood cell strain, it is only the cell in the end-stage of maturation that regularly shows appreciable and visible quantitative increase under conditions of stimulation, not the precursor cells.*

If this statement is accepted, it follows that there is no valid reason to discount the distinct possibility that leukemias of all cell types are primarily diseases of the reticulo-endothelial system. The case studies cited in the foregoing part of this paper offer some evidence that under an unknown type of stimulus to the reticulo-endothelial system (as indicated by the advent of monocytic leukemia at some phase of the disease), there may be formed large numbers of myeloblasts, lymphoblasts and even megaloblasts. It is possible in the cited cases that hyperplasia of one or another of the types of cells occurred because maturative substance for that

cell type was temporarily deficient. Eventually, the body resources were able to mobilize adequate quantities of maturing substances for this cell type, the final failure coming in inability to supply sufficient cell maturing factor for monocytes, thus in the end determining the death of the individual from monocytic leukemia.

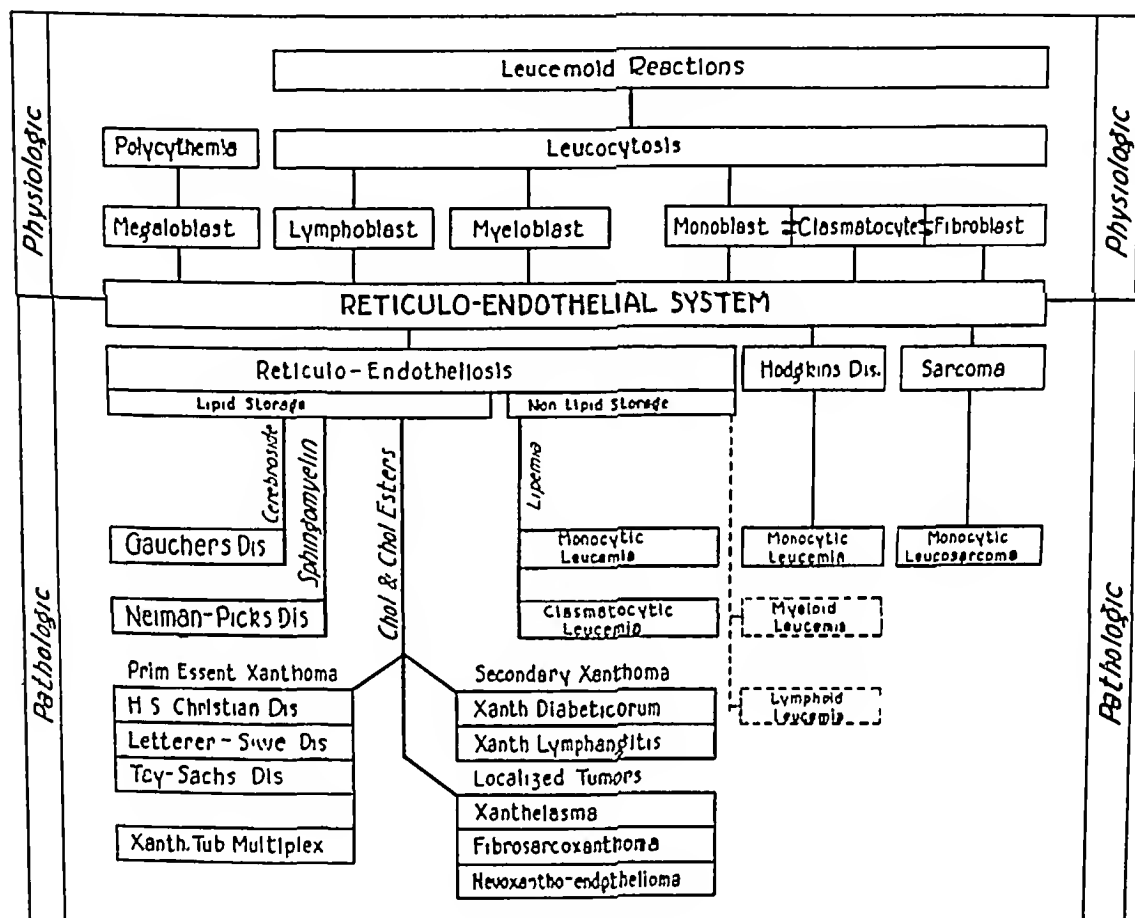


FIG 8 Diagrammatic representation of the relationship between the reticulo-endothelial system, normal blood formation (upper one-third of chart) and pathologic reactions producing disease (lower two-thirds of chart). This chart expresses the view that neoplasm of the reticulo-endothelial cells (labeled sarcoma) gives a different reaction than that of the bulk of the diseases of the reticulo-endothelial system (labeled reticulo-endotheliosis), the latter being characterized by, among other things, disturbances in lipid metabolism. Hodgkin's disease is shown as a third form of disturbance of reticulo-endothelial tissue. It is possible that sarcoidosis (not shown) is a fourth variety of reticulo-endothelial disease. Myeloid and lymphoid leukemia are indicated in broken lines as possibly also a primary disease of reticulo-endothelial cells as suggested in text.

This discussion leads to the following suggestions of a possible mechanism for the production of leukemia:

1. Leukemia, irrespective of cell type, may be the result of unknown stimulatory effects upon the reticulo-endothelial system.

2. The type of leukemia observed may be determined by the failure of the body to supply specific maturative substance or substances at one or more phases in development in that cell strain in quantity to keep up with the particular intensity

of reticulo-endothelial stimulation operating at that time in that individual organism

3 Specific cytologic maturative substances may be multiple in types and chemical identity and failure of supply of one type may not necessarily prejudice adequate supplies of another type "The chain breaks at its weakest link"

4 The bizarre varieties of leukemia regularly seen in all hematologic laboratories may result from multiple mixed failures of maturation factor varying as to type specificity and as to degree

This explanation of the production of leukemia, although admittedly speculative, satisfactorily accounts for many if not all of the puzzling features regularly encountered in patients with this disease by using only one basic mechanism without recourse to multiple theories. The concept of specific cell maturation substances is cytologically correct and the existence of one such substance for megaloblasts has been proved. In addition to the need for more information relating to other maturative factors, more facts are needed with respect to influences that are stimulatory to the reticulo-endothelial system. Particularly, in this regard is there need for additional study of lipid metabolism and the influence of lipids upon this system of cells<sup>15-18-20</sup> (fig. 8)

#### SUMMARY

1 Evidence is given from case study of reticulo-endothelial disease supporting the concept that monocytic leukemia is one form of reticulo-endotheliosis

2 On the basis of varied types of cell reactions seen in monocytic leukemia, it is suggested that all forms of leukemia may be hematologic varieties of reticulo-endotheliosis

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# THE POSSIBILITY OF PRECIPITATING THE LEUKEMIC STATE BY EMOTIONAL FACTORS

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**L** EUKEMIA in the human individual may develop without any known precursor, but frequently it occurs following a precipitating incident or set of incidents. It is apparent from a review of the literature that various physical, chemical and possibly infectious agents are involved in the inception of some of these diseases.<sup>1</sup> Exposure to irradiation from x-ray or radium, contact with or exposure to benzol or its derivatives, physical trauma, and the use of arsenical and sulphonamide drugs are some of such agents. Infections which seem to have played a part in precipitating these diseases are tuberculosis, syphilis and pneumonia. The relation of infection in this regard is not as well established as that of the physical and chemical agents. Exposure to benzol and exposure to irradiation from x-ray or radium are more clearly related to the precipitation of the leukemic state than is exposure to other agents.

No one believes that these agents cause leukemia, but it is possible that each may act to upset the normal balance of blood formation so that leukemia results.

Results of experimental work<sup>2 3 4</sup> point to the normal control of blood formation by hormonal as well as dietary factors. If leukemia is precipitated by these chemical and physical agents, this probably is brought about by changing the hormonal balance. In those cases of leukemia in which such agents are not uncovered in the pre-leukemic history, it is possible that emotional factors may have acted in a similar role in precipitating the leukemic state.

For many years, the influence of emotional reactions on physiologic processes has been well known, but only within comparatively recent times have studies revealed the significance of emotional factors in precipitating or aggravating a number of disease processes such as peptic ulcer, asthma, mucous colitis, etc. As the etiologic importance of these factors has been demonstrated, a much better understanding of these illnesses has been brought about. Milkorath<sup>5</sup> and his co-workers showed that leukocytosis, with a normal differential, "appeared to be intimately related to the psychopathologic emotion." They explained the elevation of the total white cell count on the basis of a "redistribution of the white cells from the organ reservoirs." It is well known that there is an intimate interrelationship between emotional factors, the autonomic nervous system and the endocrine glands. These findings, therefore, have led us to believe that the frequent occurrence of emotional difficulties in patients with leukemia may be more than a coincidental finding. Often these patients volunteered a great deal of material concerning their own psychologic difficulties. Therefore, we have endeavored in a few cases of leukemia to determine the emotional background, and in this paper we wish to present and discuss the emotional histories of 6 cases prior to the development of leukemia.

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brought up on a farm but at the age of 21 he was restless and ambitious to go into business. He married at 20 and his wife was never well. At 22, he worried over financial losses incurred in a filling station which he owned. His wife died, four years after their marriage, while giving birth to a child. He was worried and grief-stricken but he worked hard and did fairly well in business, saving about \$20,000 up to 1929. From 1930 through 1933 he was worried about finances most of the time. He lost his business, spent his entire savings and then went back to work for his father on a fruit farm. There he made no money, was depressed and later fought with his father and left the farm discouraged and unhappy. Throughout this entire time, he was physically well. In 1934, he again tried to go into business, determined to make good, and throughout 1934-7 he was not so easily worried and his finances were a little better managed. In 1937, he had his teeth extracted and he bled considerably and this again worried him. He also tired easily at this time and had no pep. In 1938, he used up his savings again, was unable to work well and everything then seemed to go from bad to worse. He was afraid, and seemed to have lost his nerve, and he cried easily. Late in 1938, the diagnosis of chronic myeloid leukemia was made. This patient died at home, May 1940. A necropsy was not obtained.

*Case 4* G. P. was a 46 year old man who was first seen in the Jefferson Hospital, Philadelphia, clinic the winter of 1940. He had had chronic myeloid leukemia for six years, and had been treated in several other clinics. A psychiatric interview was held in October 1940. He was of German-American extraction. His school work had been of good quality but he went to work immediately following graduation from high school. At about 25 years of age he went to work for a rubber company and a year later was married, but almost immediately afterwards he was transferred from New Jersey to a plant in Canada. Here he was placed in complete charge of construction, production and sales of a new branch of his company. His wife, however, remained in the United States the first year. Almost the entire burden of responsibility of the new plant was carried by him. He had few friends and almost no one to whom he could confide his difficulties. He was under considerable tension and felt that the responsibility was very great. At this time he began to have queer sensations in his stomach and his digestion was poor. He became easily fatigued and had great difficulty in sleeping. A little later he began to have attacks of vomiting and severe headaches. These symptoms lasted for several years and were pronounced whenever the pressure of business was heaviest.

His first child was born when he was 34. At the age of 37, or two years before leukemia was apparent, his branch of the company lost \$70,000 during the year. This was an added source of anxiety to him and he took the responsibility for the loss. His second child was born four months before the diagnosis of leukemia was made. At this time, he was overworked and worried because of the company finances as well as his own. He developed an infection of his neck and during the care of this, leukemia was discovered.

Anxiety and worry concerning his family and company were not lessened with the diagnosis of leukemia but he became philosophic. He said that he believed there were spontaneous remissions and that he would have one. He read much concerning leukemia and he tried to help in caring for himself.

He stated that he had had definite mood swings all his life, some weeks he felt on top of the world, other weeks he was down in the dumps and every little job looked twice as big as it ought to. His leukemia was discovered in December 1933 and continued through March 1941, or over seven years. He died in Memorial Hospital, New York City, March 1941, and a necropsy revealed typical findings of chronic myeloid leukemia.

*Case 5* P. H. was a 53 year old white man. He entered Lakeside Hospital, Cleveland, in the winter of 1939 where a diagnosis of chronic myeloid leukemia was made. In February 1939, a psychiatric interview was obtained. The patient had worried over small things his entire life. In 1918 he worried himself sick about everything. He had been a caretaker on an estate and he had worried about his duties, he did not believe he was doing well and everything seemed to go wrong. He became depressed and could not sleep and he lost his appetite. The estate was then sold and he became caretaker on another. The owner committed suicide, but before this happened he had felt better and was doing rather well. The suicide was a shock to him. Following this episode he had worked on yet another estate for a period of eight years. While working on this estate, he was worried and sensitive to criticism. During this time, his father died and he became depressed. He worked night and day but seemed to be in a rut. He again changed his position and became even more worried. At this time he believed someone was poisoning the

pheasants he was raising and thought that this was being done to change the control of the estate. This led to a fight with another man on the estate.

In August 1938, he became extremely depressed and wanted to die. He thought people were making fun of him and believed that someone had it in for him and was plotting against him. He thought that "the world was wrong." Then he lost interest in everything and could neither eat nor sleep. He lost his self confidence, and was always afraid he would make mistakes. Although he had death wishes, he had no suicidal ideas. He cried a great deal, and he tried to be alone and would walk the streets. His depression grew worse until, in November 1938, he was physically sick. At this time he had pains in both hips and numbness and tingling from knees to feet. He had no vitality or energy and felt a pressure on top of his head and pain in the back of his neck. He thought he was going to die and made a will and just waited. In February 1939, it was found that his spleen was enlarged and that his leukocyte count was elevated. The patient died in Lakeside Hospital, August 1941. The necropsy findings were typical of chronic myeloid leukemia.

*Case 6 M. A.*, a 40 year old white woman, entered Jefferson Hospital, Philadelphia, in December 1946. A psychiatric interview was held in January 1947. The patient appeared pale, underweight and considerably older than the age of 40. She wept when she talked about her husband. She is married to a miner and has nine children. Her husband is an alcoholic who has been cruel and a poor provider. After twenty years of marital difficulty, she finally forced her husband to leave their home in October 1945. At present she has \$93 a month from Mother's Relief and a few dollars irregularly from her husband to care for her nine children.

She was one of four children. Her father was an alcoholic and he was unstable. She stopped school at the age of 14—the 6th grade—because she had to work and help support the family. Despite her difficult life there were few somatic complaints until two years ago when she began to be easily fatigued and lost weight. It was at this time that the difficulty with her husband became acute.

She is a Catholic and very religious. The impression of the interviewer was life-long insecurity related to difficult environment and a lack of affection as a child. Her main defense was compliance, religion, patience and acceptance. It is difficult to evaluate the exact relationship between her emotional difficulties and her present disease, but it is apparent that the emotional difficulties have played a part in the development of the organic disease.

It was found in October 1946 that her spleen was enlarged and the leukocyte count was elevated. She is now being given treatment for chronic myeloid leukemia.

## DISCUSSION

In this short series, only cases of chronic myeloid leukemia have been included. Psychiatric interviews, however, have been held with 3 patients with chronic lymphoid leukemia and only one of these has given much evidence of emotional difficulties in the background. One patient with chronic eosinophilic leukemia was also interviewed, and it was found that following an accident in the plant in which he worked he frequently had somatic symptoms when he was in the room in which the accident had occurred. Otherwise, there was little in his pre-leukemic history which might have acted to precipitate the disease.

Psychiatric interviews have not been held with patients with acute leukemia, nor have we examined the emotional background of either patients or parents of any of the childhood leukemias.

Clinical data of all types have been left out of this report because each case represented a typical picture of chronic myeloid leukemia. In only one of these patients, Case 4, was there any evidence in the pre-leukemic history of chemical or physical agents which might have precipitated the leukemia. This man worked in a rubber plant but he was not exposed to chemical agents.

Anxiety, depression and chronic worry affected 4 of the 6 patients of whom

case reports are given. Each of these 4 had conversion symptoms such as nausea, vomiting and loss of appetite. One had pain in his hips and a feeling of pressure at the top of his head. Three of these 4 had loss of appetite and sleeplessness. Three of these 4 cried easily so that it might be said that each of the 4 was somewhat emotionally unstable prior to the development of leukemia. One of the other 2 had had emotional strain as a girl and later hysteria, difficulties and disappointments because she married a man she did not wholly love. The sixth had led a life of misery as a child and for twenty married years. It may not be entirely convincing that the material in such histories has precipitated the leukemic state. Heuper<sup>1</sup> has stated that the leukemic state has not been precipitated by emotional or psychic trauma. These histories, however, run somewhat contra to this statement.

Recently, it has been shown that in the past forty-five years there has been a marked increase in the incidence of leukemia.<sup>5</sup> Drugs, industrial hazards, irradiation from x-ray and atomic sources, and the increased hazard of physical trauma of our age may have brought about this increase. The increased tempo of life in the past forty-five years, with its increased sources of anxiety, worry and emotional stress and strain, may also have aided in increasing the number of deaths from leukemia.

It seems to us that all cases of leukemia in adults should be studied for any and all factors which may have played a part in the precipitation of the disease and such a study should include emotional factors. Against a statistical study of emotional factors in all cases, there should be plotted a particular study of those cases in which other factors have been ruled out.

#### SUMMARY

The emotional background of 6 cases of chronic myeloid leukemia are reviewed after psychiatric interviews. In 4, emotional instability was of long standing and in these 4, chronic worry and anxiety seemed to be an integral part of the pre-leukemic history. One of the other 2 suffered emotional trauma early in life and one had lived a life of misery as a child and an adult. Further study of emotional factors in relation to pre-leukemic histories should be made.

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# THE PRESENT POSITION IN THE TREATMENT OF CHRONIC MYELOID LEUKEMIA

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IT IS not proposed to devote this article to a detailed description of the various methods of treating chronic myeloid leukemia or to a statistical assessment of the results. The aim is rather to consider the theoretic bases on which the different forms of therapy have been devised, while, incidentally, indicating whether the observed results are such as to support the hypotheses.

That the problem of the etiology of the leukemias is almost as obscure as it was a century ago, when Hughes Bennett and Virchow, independently of one another, published the first accounts of the disease, is indisputable. And this assertion is not invalidated by the discovery of the transmissible leukemias of lower animals, which has, in fact, complicated, rather than clarified, the problem of the disease as it is observed in man. But in spite of the distressing lacuna in our knowledge, research into the treatment of the chronic forms of leukemia has been pursued in a manner, which has, on the whole, been scientific, rather than purely empiric. And this has been possible because we are less abysmally ignorant of the underlying pathologic and cytogenetic factors than we are of the causative ones.

For instance, it is patent that, in the leukemias, there is gross overgrowth of hemopoietic tissue, which in the case of our present subject of discussion—chronic myeloid leukemia—is the bone marrow. And, at this point, it is important to realize that the intense proliferation of white cells in the marrow is not to be considered as an example of hyperplasia, because that concept includes that of increase in the number of normal cells in normal arrangement, with, as a result, increased, but essentially normal, functional ability. Thus, the extremely active cellular marrow found in many infections is an example of hyperplasia, whereas the more or less disorderly proliferation in myeloid leukemia is to be considered as pathologic overgrowth. And this is true whether the malady be included with the neoplastic ones or not.

Now, in normal postnatal life, the mature granulocytes found in the circulation arise from mitotic division of granular myelocytes. That is to say, the polymorphonuclear leukocytes, with their specific granulation, arise from simpler cells, which do, however, already possess characteristic granules in their cytoplasm. Less mature cells—myeloblasts—are not involved in the process, forming, as it were, a reserve of stem-cells which are not normally called into action, and, even in severe infections, the marrow contains few myeloblasts, whereas myelocytes are very numerous and show signs of active proliferation. In fact, it is probably true that extension of the genealogic tree back to the myeloblast stage results in the formation of abnormal granulocytes. In other words, there is reason to suppose that the abnormal white-cells which are so prominent a feature of the blood and marrow in chronic myeloid leukemia are produced from maturation of myelo-

blasts, and this view is consistent with the generally accepted statement that the outlook is the worse the greater the proportion of myeloblasts in the marrow.

Deterioration, that is to say, a tendency towards acuteness, in chronic myeloid leukemia is detectable in the marrow earlier than in the blood, because there is great overgrowth of myeloblasts (at the expense of myelocytes) *before* there is any noteworthy change from the "chronic" type of blood picture. It is, therefore, reasonable to assume that conversion of chronic into acute leukemia comes about by a peculiar process of dedifferentiation.

First, there is increase in the number of myeloblasts in the marrow, although some or many of these cells have the power of maturing into more or less normal myelocytes, which, in turn, may give rise to polymorphonuclears or they may themselves emerge into the circulation. Secondly, there is still further deterioration of the functional activity of the myeloblasts, which lose their power of becoming differentiated into myelocytes, and this represents the stage of complete and irreversible conversion of chronic myeloid leukemia into the acute form. Of course, intermediate phases are well known, as witness the presence of ill-formed myelocytes in the peripheral blood.

The brief discussion above will serve as a preface to the more distinctly therapeutic problems which confront us, while, later, a rather more recondite consideration of the cytologic factors will be required as an introduction to the most modern methods of treatment.

Probably the oldest method of treating chronic myeloid leukemia is by administration of arsenic, and this is especially interesting as an example of more or less successful empiricism, because the mode of action of the drug in blood dyscrasias is still obscure. Now, of two facts, there can be no dispute: first, arsenic, properly exhibited, can bring about clinical and hematologic remission in many cases, and, secondly, that this is brought about by decreasing the activity of myeloblasts in reproducing themselves and in giving rise to myelocytes. In other words, arsenic, at least for a time, is able to cause leukocytopoiesis to proceed along more or less normal lines, while, at the same time, decreasing the gross overactivity of myelocytes in dividing and in differentiating into polymorphonuclears.

Physicians of a past generation, who, like us, were not averse to hiding ignorance under a cloak of words, valued arsenic for what was known as its "alterative" action. And it is indisputable that this term is admirably descriptive of the effect of arsenic in chronic myeloid leukemia. This was equally well shown, before the introduction of liver treatment, in pernicious anemia, by the effects on the erythrocyte picture.

There are many points of practical importance in connection with arsenical medication, as Forkner and Scott emphasized in 1931, when the drug, which had almost fallen into disuse in the treatment of leukemia, was given a new lease of usefulness.

A few of the advantages may be mentioned here. Thus, arsenic is cheap and easily available, and, if given in the early stages of chronic myeloid leukemia, it usually produces a good remission, which can sometimes be maintained for months or even years by continuing to administer maintenance doses. This second feature is a great

advantage over irradiation, which has, of course, to be interrupted when the blood picture is more or less normal, as, otherwise, aplasia of the marrow may ensue

Not only does arsenic cause a reversion of the leukocyte picture to a more normal composition, but it produces amelioration of the anemia. But it is debatable whether this is the result of a direct action of erythropoiesis, whether it is due to relief of pressure on the red stem-cells, or whether both (and perhaps other factors also) play a part

This is not the place to describe the minutiae of therapeutics, but it is well to point out that, when arsenic will no longer maintain the patient in a state of remission, irradiation may still do so. However, it is essential to recollect that exposure to therapeutic irradiation shortly after a prolonged course of arsenic is likely to cause more severe reactions than would a similar dose of x-rays in a patient who has not had the drug. This is alleged to be due to secondary radiation from the arsenic stored in the tissues, but whatever the explanation, a history of having taken arsenic for months before starting radiation treatment indicates the need for small and experimental dosage of x-rays

Conversely, arsenic may be effective in some cases which have become resistant to x-rays, still inducing a remission when irradiation will no longer do so. This is, of course, true only when the blood and marrow are still characteristic of the chronic form of the disease, but, if the failure of x-rays is due to conversion into the acute (myeloblastic) state, no known treatment will bring about a remission

Another method of medicinal treatment, which, like arsenic, has to some extent fallen into undeserved disuse, is benzol, which also has the advantage of being given by mouth, while the dangers which have been attributed to its administration are entirely the result of gross overdosage

Benzol is a well-known marrow poison, which has caused a good deal of trouble in various industrial processes for this reason. It appears to damage the platelets, the granulocytes and the red corpuscles in that order, affecting the parent-cells and so reducing the mature forms in the blood

It was this knowledge of the action of benzol which led to its use in treating leukemia, but there is no doubt that its effects are not entirely due to its myelotoxic action. If given in suitable doses, the leukocyte count falls as a result of decrease in the number of immature and abnormal white-cells, while the red count rises, and the changes in the marrow are of the same type, viz., decrease of myeloblasts and a more normal activity of the myelocytes. In fact, a new investigation of benzol is overdue, because it has now been established that prolonged exposure of healthy persons to small doses of benzol greatly increases the likelihood of the development of chronic myeloid leukemia

If benzol is given when the leukocyte count is very high, it is best exhibited in capsules with olive oil, each containing 0.5 Gm. of benzol. The course is started with four capsules daily, and, if there is no nausea, is rapidly increased to a maximum of eight or ten daily. This dose is continued until the leukocytes have fallen to about 50,000 per cu. mm., when the dose is gradually reduced until the white-cell count is about 15,000 per cu. mm. Then, it is often possible to find a suitable maintenance dose, usually in the region of 0.5 Gm. two or three times a week



Again like arsenic, benzol is still effective when radiation has become ineffective, but, unlike arsenic, a preliminary course of benzol is not liable to cause trouble during subsequent radiation treatment.

Benzol acts as an essentially destructive agent, which has a rather more powerful effect on immature cells than on mature ones; hence, the beneficial results in chronic myeloid leukemia. Unlike radioactivity and urethane, its effect on dividing cells is no greater than on resting ones, so that it cannot be regarded as affecting the essential abnormality which is the cytologic basis of the disease.

For many years, the usual method of treating chronic myeloid leukemia has been with x-rays, and, as the results obtained are rapid and spectacular, arsenic and benzol have fallen into a poor second place. As already indicated, the dislike of these two drugs is an ill-founded one.

Deep x-ray therapy can rapidly bring about a remission in the great majority of cases of chronic myeloid leukemia, but that is no reason for assuming that the radiologist is the right person to have charge in such cases. The disease lies in the province of the physician, who is in a much better position than is the specialist radiologist to determine the most suitable therapy and to assess the results in individual cases.

This is not a denial of the right of the radiologist to determine which particular technic of irradiation is to be employed, but, despite quite acrimonious divergences of opinion between different schools, it can truthfully be said that the results obtained by the different methods are all approximately the same. The length of the remission which is brought about is no longer when one radiologic technic, rather than another, is employed, and the development of radioresistance is not accelerated or postponed by any particular dosage or by exposures of different areas.

It is probably no exaggeration to say that the development of therapeutic irradiation in cases of chronic myeloid leukemia has never been exploited as fully as it might have been. Most physicians have been satisfied with the remission brought about by x-ray treatment and have kept careful watch for the earliest hematologic signs of relapse, and, when these have appeared, have returned the patient to the radiologist for further treatment.

The mild contempt with which arsenic has been regarded, and the fear of benzol as a possible cause of aplastic anemia, have prevented combined treatment from being used as extensively as might have been expected from our empiric knowledge of therapeutics. In part, of course, this failure has been due to the habit of handing cases of chronic myeloid leukemia to the radiologist.

That the length of a remission which has been induced by x-rays can be prolonged by administration of maintenance doses of arsenic or benzol is indisputable, but there are no published records of individual cases which have been dealt with in this way. More and more attention has been given to irradiation, and less and less to medicinal treatment. Thus, sodium phosphate, made radioactive by the cyclotron, has been hailed as a great advance in treatment, whereas, in fact, its main advantage is that it can be administered orally, while the results have been

much the same as those of other methods of exposing the leukemic cells to the destructive action of rays

Even the most enthusiastic advocate of radiation treatment has not, it may be assumed, ever supposed that the method, however modified it might be in the future, would result in cure of leukemia. Some workers have sought for a hypothetical virus, others for a toxin, and yet others for indications of a chemical or an endocrine factor, and it is probably correct to say that more and more adherents to the chemical (or constitutional) theory are won annually, although, of course, acceptance of this view does not exclude leukemia from the neoplastic class.

Only a very few indications of the evidence can be given here, but they may suffice to stimulate further work, while they form a more or less rational basis for the further discussion of medicinal treatment. First, there is the well-known fact that the incidence of leukemia may be familial, although, admittedly, this is far from common. Nevertheless, in the case of a disease so relatively rare, the existence of such cases suggests that some intrinsic factor is of etiologic importance, and such a view is supported by the distinctly greater frequency of the chronic myeloid form in Jews. Secondly, the fact that long-continued exposure to small concentrations of benzol (and perhaps of x-rays) increases the incidence of chronic myeloid leukemia seems to demonstrate that a chemical change underlies the abnormal proliferation which characterizes the disease.

But perhaps the best evidence, indirect though it is, may be found in certain more academic observations. Thus, the cytologic changes which are accepted as being indicative of malignancy are present, in more or less well-defined form, in leukemia. The most conspicuous of these changes are hyperchromatism which is due to increase in the size of the chromosomes, enlargement of the nucleolus, variation in the number of chromosomes, defects in the spindle during mitosis, and increased variability in the size of the cells and the nuclei. Obviously, the question whether there is any casual sequence among these cell abnormalities demands answer.

An inadequate, but, I hope accurate, review of the fundamental work of Caspersson, Darlington, Claude, and Thomas, will give some indication of the present position of the problem. Thus, it is known that nucleic acid and nucleoproteins play an outstanding part in cellular activities. Ribose nucleic acid is produced by the heterochromatic regions of the chromosomes, and is found in the nucleoli and in the cytoplasm. It is closely associated with the synthesis of the self-perpetuating proteins in the cell-body. Desoxyribose nucleic acid, which is produced during the prophase of mitosis, becomes attached to the chromosomes and is responsible for their reproduction. While this is happening, the nucleolus, with its store of ribose nucleic acid, dissolves and disappears. Then, when the chromosomes have divided and have reformed as daughter nuclei, their charge of desoxyribose nucleic acid is given up and is reconstituted as ribose nucleic acid in the freshly formed nucleoli.

In normal cells, the two nucleic acids are so balanced that chromosome reproduction and cytoplasmic synthesis are balanced, probably by the regulating action of heterochromatin. If this be so, increase of heterochromatin will lead to excessive nuclear synthesis and therefore, to an increased rate of nuclear division.

Endless and rather fruitless speculation along these lines is possible, but, even without departing far from solid experimental observations, something of importance can be inferred. For instance, Beadle showed that mutation in a single nuclear gene can induce polymitosis, but the observations briefly discussed above indicate that mutation in cytoplasmic elements may also cause great change of cellular characters. Then again, the existence of self-perpetuating elements in the cytoplasm (the plasmagenes of Darlington) throws some light on the characters of "viruses," because both plasmagenes and viruses depend for their continuance on a chemical equilibrium, and Potter alleged that the "cancer virus" is almost identical with an enzyme X, which is a complex of respiratory enzymes of the nature of a ribonucleoprotein.

The chemical and the virus theories of the origin of malignant conditions, among which leukemia must be included (at least on cytologic grounds), are thus found to be almost, if not entirely, unified.

These observations and similar reflections, together with further experimental work, form the basis of the urethane treatment of leukemia introduced by Haddow and Sexton, and investigated, by Paterson, Haddow and others. And one point that emerges very clearly is that nucleic acids are of outstanding significance in the leukemias (and probably in all forms of malignancy). This is strikingly shown by the action of colchicine, which was first employed in medicine for its action in gout (a malady in which the metabolism of purins is upset), was then observed to act on the bone-marrow, later was discovered to have a remarkable effect on mitosis (for instance, in inducing polymitosis in wheat), and is now known to have some effect in leukemias. And when the fairly common concomitance of gout and chronic myeloid leukemia is recollected, the skein of evidence, incriminating the purins, although still unravelled, seems to be fairly complete.

During experiments on the growth-inhibiting effects of urethane on animal tumors, Haddow and Sexton observed striking changes in the cells of the Walker rat carcinoma and a fall of the leukocyte count in some cases, and as there is little evidence that urethane is liable to cause aplasia of the marrow, it is preferable to benzol while being more efficacious than arsenic. This suggested the trial of the drug in leukemias, and it is not too much to assert that further therapeutic application of this substance has shown that it is probably the most satisfactory treatment for chronic myeloid leukemia now available.

Urethane remains effective when x-rays have failed, but seems to be the method of treatment indicated from the outset in most cases. But it is not to be supposed that the drug is in fact a cure for the disease.

The composition of the leukocyte-picture approaches normal, the red cells and hemoglobin rise, and the spleen retires behind the costal margin. And it appears that these results depend upon the action of urethane at the prophase of mitosis of the least differentiated stem-cells of the blood. There is, in fact, a redistribution of the nucleic acids, as shown by the Feulgen reaction. The way is thus open for the micro-chemist to find a new weapon for the even more fundamental treatment of the leukemias, but already urethane can often produce a prolonged period of clinically perfect remission.

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# THE USE OF URETHANE (ETHYL CARBAMATE) IN THE TREATMENT OF LEUKEMIA

## A PRELIMINARY REPORT

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HADDOW and Sexton<sup>1</sup> noted a coincidental depression of the leukocyte count during the course of observations on the effect of various forms of urethane on the growth of experimentally produced tumors in the rat. As a result, the various forms of urethane, principally ethyl carbamate, were subsequently utilized in the treatment of leukemias and other types of malignant disease by Paterson, Thomas, Haddow and Watkinson.<sup>2</sup> Encouraging results followed the administration of urethane in the majority of 13 cases of myelogenous leukemia and, to a lesser extent, in 9 cases of lymphatic leukemia. The most favorable response consisted of a reduction of the total leukocyte count to normal levels, production of a more nearly normal differential pattern, elevation of hemoglobin levels and diminution in the size of enlarged lymph nodes or spleen. These effects were sustained for variable periods, the longest period of observation recorded being eleven months. Symptoms of gastrointestinal irritation of mild to moderate degree rather commonly followed administration of the drug. Aplastic anemia developed in 2 cases, in one of which there was a fatal termination. In general, the results obtained were regarded as much like those following the use of the standard method of irradiation therapy.

The mode of action of urethane remains obscure. Kirschbaum and Lu<sup>3</sup> observed that the administration of a single anesthetic dose of urethane to mice with myelogenous leukemia resulted in a drop, within twenty-four hours, in the total leukocyte count and in the appearance of many mature cells in the bone marrow. The number of mitotic figures in the myeloid cells of the marrow was decreased and it was suggested that maturation may have been secondary to inhibition of mitosis in blast cells. Johnson<sup>4</sup> observed that urethane exerts an antisulfanilamide effect, similar to that of para-aminobenzoic acid, in work with certain strains of luminous bacteria. This would suggest that urethane's growth-inhibiting property may result from interference with utilization, in cellular metabolism, of some natural amine.

At the Mayo Clinic, clinical experience with urethane (ethyl carbamate) in the treatment of leukemic states now covers a period of approximately eight months. While the ultimate evaluation of this substance as a means of treatment for the leukemias obviously remains to be determined in the future, certain current observations may be of interest to those concerned in the management of these perplexing conditions.

## RESULTS OF TREATMENT

*Chronic myelogenous leukemia* In 14 cases, urethane is now being used as the sole means of treatment. The patients in these cases have been under treatment for periods ranging from seven weeks to eight months. In all instances, the response to treatment thus far has been regarded as satisfactory and no supplementary measures have been required. There has been a consistent reduction in the total leukocyte count (table 1) accompanied by a parallel reduction in the degree of myeloid immaturity apparent in differential analysis of smears of the peripheral blood and sternal bone marrow. The erythrocyte count and hemoglobin values have either shown a significant increase or have remained virtually unchanged. In no instance has anemia or clinically significant thrombocytopenia developed during

TABLE 1—*Early Effects of Treatment of Chronic Myelogenous Leukemia with Urethane*

Urethane, Gm	Leukocytes per cubic millimeter of blood	
	Before treatment	Six weeks (average) after treatment
65	124,000	23,500
95	234,000	93,600
105	155,000	18,600
68	163,000	21,100
87	145,000	25,600
110	307,000	26,000
91	147,000	46,000
75	260,000	13,000
40	137,000	9,590
52	130,000	23,400
168	210,000	84,000
21	164,000	8,300
85	118,000	14,750
72	117,000	42,100

the course of treatment. Splenomegaly has been consistently and rapidly diminished in degree, although in no case has a previously palpable spleen disappeared entirely. The degree of response apparently was not affected by the initial level of the total leukocyte count, the duration of the illness or the amount of previous irradiation therapy.

A satisfactory response to treatment was achieved in three to ten weeks (average six weeks) following the administration of 21 to 168 Gm of urethane (average 80 Gm). The drug was usually administered in a dosage of 1 Gm three times a day at the outset. This amount was reduced to 1 to 2 Gm daily as the leukocyte count descended. The primary fall in the leukocyte count usually occurred seven to fourteen days after the institution of therapy and was often preceded by a transient elevation. However, in 2 cases, twenty-one to twenty-eight days of treatment was required to produce the initial depression of the leukocyte count.

The dosage necessary to maintain the leukocyte count at relatively normal

levels (less than 20,000 per cu mm of blood) has been highly variable, although for the 2 patients under observation for the longest periods, 0.5 to 1.0 Gm daily has been found adequate. Weekly leukocyte counts are necessary in determining the long-term needs in the individual case, the amount of urethane administered being increased or reduced accordingly. An effort has been made to maintain the total leukocyte count at 20,000 or less per cu mm and the drug has been temporarily discontinued when the total leukocyte count has been less than 10,000 per cu mm. No cases of chronic leukopenic myelogenous leukemia were included in this series.

On cessation of therapy, recurrence of leukocytosis with myeloid immaturity invariably occurred after variable periods of time.

Mildly to moderately severe symptoms of gastrointestinal irritation occurred in approximately one third of the cases but disappeared with continued administration of the drug, although a reduction in dosage was sometimes necessary. A direct relationship between the size of the daily dose and the frequency of gastrointestinal complaints was soon established and, in recent months, an amount in excess of 3 Gm daily has rarely been prescribed. No other toxic manifestations attributable to the drug were observed in this series.

*Acute myelogenous leukemia* Urethane was administered in 2 cases for periods of ten to fourteen days with no significant change in the clinical course, although there was some decrease in the degree of myeloid immaturity in smears of the peripheral blood and sternal marrow.

*Chronic lymphatic leukemia* The drug was administered, in apparently insufficient amounts, in 2 cases. There was no appreciable effect on the total leukocyte count.

*Acute lymphatic leukemia* Urethane has been administered for periods of one to three weeks in 8 cases. In three instances, there was a precipitous fall in the total leukocyte count, but in none was the general clinical course materially influenced.

*Acute monocytic leukemia* In 2 cases, treatment of one to three weeks' duration produced no measurable hematologic or clinical change.

#### COMMENT

Our experience would indicate that urethane can be expected to produce a temporary hematologic remission in cases of chronic myelogenous leukemia. This remission is similar, in superficial characteristics, to that observed after irradiation therapy. There is, at the present time, no indication that urethane offers more than other agents which are used palliatively in this disease. However, by virtue of its convenience of administration and the possible advantage of controlled, continuous action, urethane may be found preferable to other methods of treatment in use at the present time.

We have had insufficient experience with the use of urethane in the treatment of chronic lymphatic leukemia to warrant an opinion as to its efficacy. However, the reported experiences of Paterson and co-workers<sup>2</sup> would seem to justify continued use of the substance in such cases.

While, like irradiation therapy, urethane may produce a rapid decrease in the number of immature leukocytes circulating in the blood in some cases of acute

myelogenous and lymphatic leukemia, there is no evidence to suggest that the usual course of these conditions has been beneficially influenced

Despite the absence of serious toxic effects encountered in this series to date, the potential production of aplastic anemia and other complications<sup>5</sup> by this substance must be kept in mind

#### SUMMARY

Limited experience with the use of urethane in the treatment of leukemia indicates that this substance presents a considerable promise as a palliative agent in chronic myelogenous leukemia. It has no apparent value in the treatment of acute leukemia.

Further extensive observation will be necessary to provide a true measure of the clinical usefulness of this preparation.

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## URETHANE THERAPY IN LEUKEMIA

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THE purpose of this paper is to report the results of urethane therapy of the leukemias, based on a study of 24 patients

In April, 1946, Haddow and Sexton<sup>1</sup> described the influence of various carbamic esters on experimental rat cancers. Ethyl carbamate (urethane) yielded the best results. It alone produced inhibition of tumor growth and fibrous replacement of the cancerous rat tissue. For this reason, and since urethane is relatively nontoxic for humans and is easily obtainable, the experiments were transferred to human subjects with carcinoma of the breast and other malignancies. The results were generally disappointing. It was, however, noted that a few of these patients developed leukopenia while taking urethane. This observation motivated, in 1943, the first clinical trial of the effects of urethane in leukemia and allied disorders.

In May, 1946, Paterson, Haddow, Thomas, and Watkinson<sup>2</sup> reported the results of urethane treatment of 32 leukemic patients. The drug was administered orally in an average dose of 3 to 4 grams daily. The drug proved "effective" in approximately one-third of the patients, producing in this "favorable group" reduction in size of enlarged spleens and of lymph nodes and causing reversion of the blood picture to more normal values. These workers found the urethane effect to be approximately equal in value to that of standard deep x-ray therapy in a control series of similar cases. Of their 32 urethane-treated patients, 19 had myelogenous leukemia, and 8 of these were benefited. Clinical and hematologic remissions were maintained for periods of 2 to 6 months. Of 13 patients with lymphatic leukemia, 2 responded similarly. The remaining 22 were either partially improved, could not tolerate the drug, or died during treatment.

Toxic side effects observed by the British workers included nausea, drowsiness, anorexia, diarrhea, and suggestive evidence of marrow hypoplasia.

On the basis of these results, parallel observations were started at the Hospital of The University of Pennsylvania in June, 1946. At the time of this writing (September, 1947) we have treated a total of 27 patients suffering from leukemia. Three of these are too recently treated for accurate evaluation. Our report is based on the remaining 24.

*Dosage and methods of administration.* The usual dose of urethane was 4 grams (range 2 to 6 Gm) daily, given in solution, orally. A mixture similar to that used by the British group was employed in most of the patients.

Urethane	30
Syrup of orange	50
Chloroform water to	300
(each 5 cc contains 0.5 grams urethane)	

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This mixture was administered (1 to 4 teaspoonsful) after each meal, in a small amount of some flavored carbonated beverage or fruit juice. In a smaller group of patients, the drug was administered in 0.5 Gm gelatin capsules. This method of administration proved to be impracticable because of the hydroscopic nature of the urethane. Rectal suppositories (each 0.5 Gm urethane in cocoa butter) were tried in 2 patients who developed nausea from oral medication. Rectal irritation occurred within a few days, requiring cessation of this method of administration.

In one patient (Case 4) with a large leukemic tumor, 50 per cent urethane ointment was employed locally, in conjunction with oral urethane medication. The ointment was prepared by melting urethane crystals and incorporating the solute in aquaphor. The leukemic tumor disappeared under this therapy, although the patient died subsequently.

Urethane intravenously has been our most recent mode of administration. Ampoules\* containing 1 or 2 Gm urethane in 20 cc of normal saline solution or distilled water, were mixed with 200 cc of normal saline and the resulting mixture was administered in 15 to 30 minutes intravenously, without untoward reactions.

*Clinical material.* The results in 24 leukemic patients are reported. Table 1 indicates the distribution of types of leukemia.

TABLE 1

Acute Leukemias	10
Myelogenous	7
Lymphatic	2
Monocytic	1
Chronic Myelogenous Leukemia	7
Chronic Lymphatic Leukemia	7

## RESULTS OF URETHANE THERAPY

### *Chronic Myelogenous Leukemia*

Our results in 7 cases of chronic myelogenous leukemia are summarized in table 2. It is to be noted that in 4 cases, urethane therapy was started during terminal stages of the disease, after resistance† to x-ray therapy had been established. Two cases are regarded as showing satisfactory clinical and hematologic remissions after urethane therapy. Case 4 is of special interest because of a successful remission maintained despite the complication of pregnancy which is now in its seventh month and is proceeding uneventfully.

### CASE REPORTS

*Case 4.* This white female, age 30, first noted weakness, nausea, and abdominal enlargement during the latter months of 1945. When first examined on December 12, 1946, she exhibited massive splenomegaly (19 cm below the left costal margin), and slight liver enlargement. Blood Hgb 8.38 Gm, WBC 320,000, immature myeloid leukocytes‡ 34 per cent. Urethane, 3 Gm daily, in solution orally, was started on December 14, 1946, and was increased to 4 Gm daily five days later. By the forty-third day of treatment,

\* Prepared according to our specifications regarding sterility, isotonicity and pH corrections by Dr F. B. Peck, Eli Lilly Laboratories.

† The term "x-ray resistance" is here employed without implications of any kind except to designate the fact that our own Department of Roentgenology, employing the techniques and dosages judged by this Department to be the best under the circumstances, had failed to obtain a remission from one or more recent courses of therapy which formerly in the same patient had produced a remission.

‡ The term "immature myeloid leukocytes" refers to the combined percentage of myelocytes, promyelocytes, and myeloblasts.

the spleen measured 11 cm and the blood count was Hgb 9 9 Gm , WBC 11,000, immature myeloid leukocytes none The drug was continued until March 22, 1947 (ninety-eighth day), when the spleen measured 5 cm and the blood showed Hgb 13 0 Gm , WBC 10,000, immature myeloid leukocytes 3 per cent The patient was symptom-free

This patient conceived while on urethane therapy When last seen (Aug 26, 1947), she was asymptomatic, vigorous, and in the seventh month of pregnancy The spleen was palpable 6 cm below the costal rim Blood Hgb 11 4 Gm , WBC 35,000, immature myeloid leukocytes 7 per cent

*Case 9* White female, age 38 Urethane was first administered during the late stage of chronic myelogenous leukemia of over six years' duration The patient had failed to respond to her most recent (seventh) course of irradiation She showed cachexia, lymphadenopathy, and an enormous spleen which al-

TABLE 2 —Chronic Myelogenous Leukemia

Case	Original Status	WBC Start U	U (grams) U (days)	WBC End U	Result
#4 W 30 F	Untreated	320,000	256 98	10,000	Good Pregnant
#9 W 38 F	Duration 6 years X-ray resistant	149,000	208 57	76,000	Died during treatment
#10 W 46 M	Duration 3 years	103,000	13 13	9,000	Relapsed in 2 weeks Died
#17 W 65 M	Duration 1 year	43,000	17 17	20,000	Stopped U because of nau- sea Died 2 mo later
#20 W 50 F	Duration 4 years X-ray and P <sub>22</sub> resistant	395,000	28 7 (I V )	220,000	Died during treatment
#22 W 63 M	Duration 2 years Fowl- er s solution	55,000	29 10 (I V )	14,000	Poor Given x-ray ther- apy Died
#24 W 51 F	Duration 4 years X-ray therapy in remission	50,000	116 44	5,100	Good

most filled the abdomen Blood Hgb 7 97 Gm , WBC 149,000, immature myeloid leukocytes 34 per cent Urethane, 4 Gm daily, in solution orally, was started on October 25, 1946, and continued until the patient expired on December 23, 1946 Blood Hgb 8 02 Gm , WBC 76,000, immature myeloid leukocytes 31 per cent

*Case 10* This white male, age 46, was a terminal, much treated case of chronic myelogenous leukemia of over three years duration He was readmitted to the hospital in an acute relapse phase of his disease Urethane, 1 Gm daily, in solution orally, was given for thirteen days with a fall in the leukocyte count from 103,000 to 9,000 No clinical improvement occurred and the patient died within two weeks after stopping the drug

*Case 17* A white male, age 65, was a terminal case of subacute myelogenous leukemia of one year duration, resistant to irradiation He was unable to tolerate an adequate dose of urethane either orally or by suppository A leukemic tumor of his forehead shrank in size and finally disappeared after application of 50 per cent urethane-aquaphor ointment He died soon thereafter

*Case 20* This white female, age 50, was a leukemic of four years duration. After numerous courses of irradiation she was finally considered x-ray resistant. She was cachectic, anasarcatous, and exhibited massive enlargement of lymph nodes, spleen, and liver. Blood Hgb 4.95 Gm, WBC 395,000, immature myeloid leukocytes 52 per cent. Urethane, 2 Gm, then 4 Gm daily, intravenously was started May 25, 1947, and continued until she died suddenly on June 1, 1947, soon after thoracentesis. On May 31, 1947, she had been clinically unimproved, although her white blood count had dropped to 22,000 on this date.

*Case 22* This white male, age 63, had suffered weakness and weight loss for three years. He had just completed a short course of Fowler's solution, when first examined on June 13, 1947. He presented fever

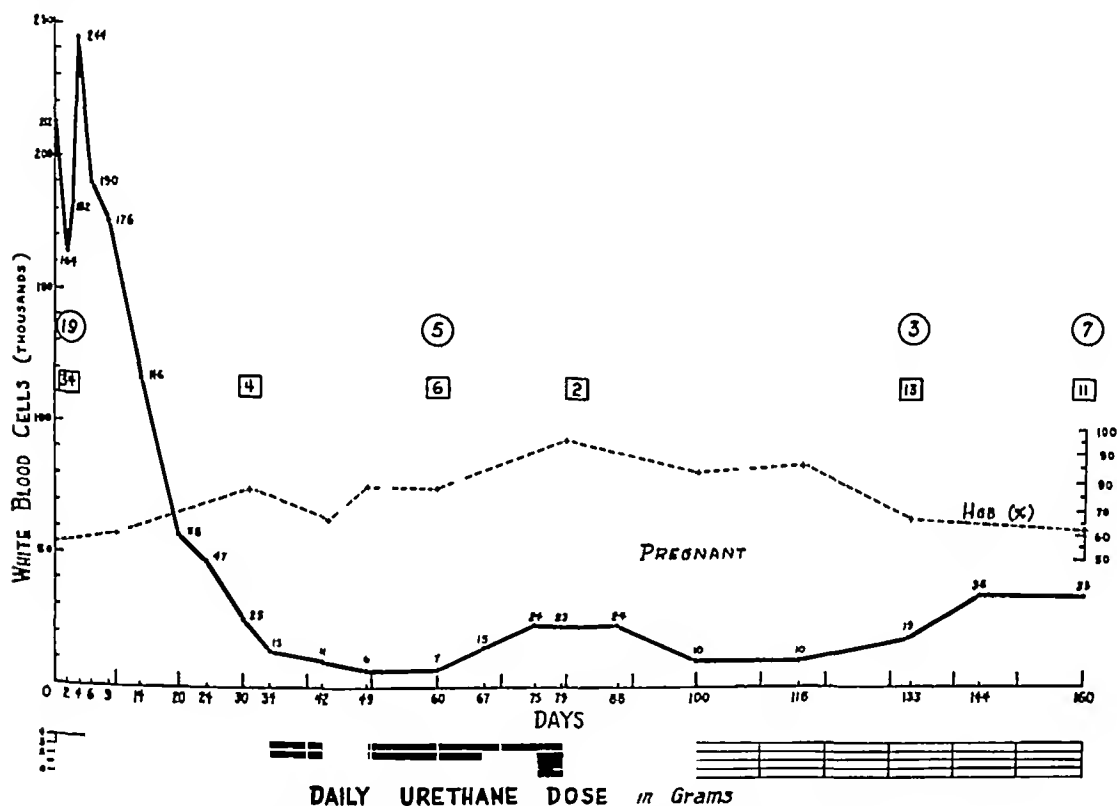


FIG 1. CASE 4, CHRONIC MYELOGENOUS LEUKEMIA IN A WHITE FEMALE, AGE 30 (□ = PER CENT BLASTS, PROMYELOCYTES, AND MYELOCYTES, ○ = SPLENIC SIZE IN CM)

pallor, petechiae, edema, and moderate splenic and lymph node enlargement. Blood Hgb 7.59 Gm, WBC 63,000, immature myeloid leukocytes 33 per cent, Platelets 45,000. Intravenous urethane 2 Gm was given on June 19, 1947 (WBC 55,000), and was increased to 3 Gm daily the following day. On June 29, 1947, the drug was stopped after a total of 29 Gm intravenously. The patient was unimproved. Blood Hgb 7.5 Gm, WBC 14,000, immature myeloid leukocytes 13 per cent. Transfusions were ineffective. Radioactive phosphorus was given, but the patient died within two weeks.

*Case 24* This white female, age 51, was known to have leukemia of four years duration. She had had several excellent x-ray induced remissions. When seen in the clinic, May 8, 1947, she complained of recent epistaxis and weakness. Her general condition seemed good. The lymph nodes and spleen were not enlarged. Blood Hgb 12.4 Gm, WBC 50,000, immature myeloid leukocytes 17 per cent, Platelets 142,000. Over the next forty-four days, a total of 116 Gm urethane, in solution orally, was administered. On June 27, 1947, she felt entirely well. Blood Hgb 13.2 Gm, WBC 5,100, immature myeloid leukocytes 2 per cent, Platelets 256,000. When last seen Aug 12, 1947, she was clinically well. Blood Hgb 13.7 Gm, WBC 26,000, immature myeloid leukocytes 10 per cent.

Chronic Lymphatic Leukemia

Our results in 7 cases of chronic lymphatic leukemia are summarized in table 3. It is of interest that the first case we treated with urethane (Case 1) has now enjoyed an entirely satisfactory remission of slightly over one year's duration.

CASE REPORTS

*Case 1* This white female, age 57, first noted weakness, and left-sided heaviness in May, 1946. When first examined by us on June 20, 1946, massive splenomegaly and slight lymphadenopathy were found. Blood Hgb 12.2 Gm, WBC 700,000, lymphocytes 96 per cent, Platelets 118,000. Urethane 2 Gm daily, in solution orally, was started on June 24, 1946, and increased to 4 Gm daily eleven days

TABLE 3 —Chronic Lymphatic Leukemia

Case	Original Status	WBC Start U	U (grams) U (days)	WBC End U	Result
#1 W 57 F	Untreated	700,000	273 72	4,500	Good Remission 362 days plus
#3 W 54 F	Duration 1 year 1 course x-ray therapy	102,000	191 76	6,400	Good Remission 184 days plus
#6 W 59 M	Untreated	58,000	683 299	21,000	Poor
#7 W 67 M	Duration 1½ years X-ray 7 months before treat- ment	233,000	225 62	8,700	Hem I Good Clin Poor Died at 75 days
#8 W 61 M	Untreated	238,000	297 63	293,000	Poor Given x-ray ther- apy Fair response
#13 W 52 M	Untreated (Leukemia Cu- tis)	44,000	521 136	26,000	Poor
#16 W 45 M	Untreated	265,000	218 63	22,000	Poor Hypoplastic mar- row

ater. Marked shrinkage in size of the spleen was apparent after forty-three days of treatment. On September 3, 1946 (seventy-second day of treatment), after a total of 283 Gm of urethane, treatment was stopped. The patient was entirely symptom free, the spleen was barely palpable. Blood Hgb 11.41 Gm, WBC 4,500, lymphocytes 53 per cent. When last seen on August 29, 1947, the patient was in good condition. The spleen again showed slight enlargement. Blood Hgb 12.4 Gm, WBC 32,000, lymphocytes 92 per cent.

*Case 3* This white female, age 54, was found to have asymptomatic chronic lymphatic leukemia in September, 1945, and a brief course of irradiation therapy was given. She was first seen in this clinic December 7, 1946. She was still asymptomatic, there was no lymphadenopathy and the spleen was moderately enlarged. Blood Hgb 12.95 Gm, WBC 102,000, lymphocytes 87 per cent. Urethane 3 Gm daily, in solution orally, was started. The drug was stopped on February 16, 1947 (seventy-sixth day of treatment), after a total dose of 191 Gm, her spleen was impalpable, she was still symptom free, and her blood count was Hgb 12.5 Gm, WBC 6,400, lymphocytes 66 per cent.

The drug was resumed on April 27, 1947, because of a rise in the leukocyte count to 19,800. On May 19, 1947, after a total dose of 69 Gm, urethane was again discontinued when her blood count was Hgb 12.0 Gm, WBC 6,200, lymphocytes 40 per cent. When last seen on August 15, 1947, the patient was still asymptomatic, her spleen was barely palpable and her blood count was Hgb 33.42 Gm, WBC 11,000, lymphocytes 79 per cent.

*Case 6* This white male, age 59, was found in a routine blood count to have 43,000 leukocytes, with 62 per cent lymphocytes. He was asymptomatic, with shotty lymphadenopathy and a barely palpable liver and spleen. Although he required no specific treatment at this time, urethane was started on July

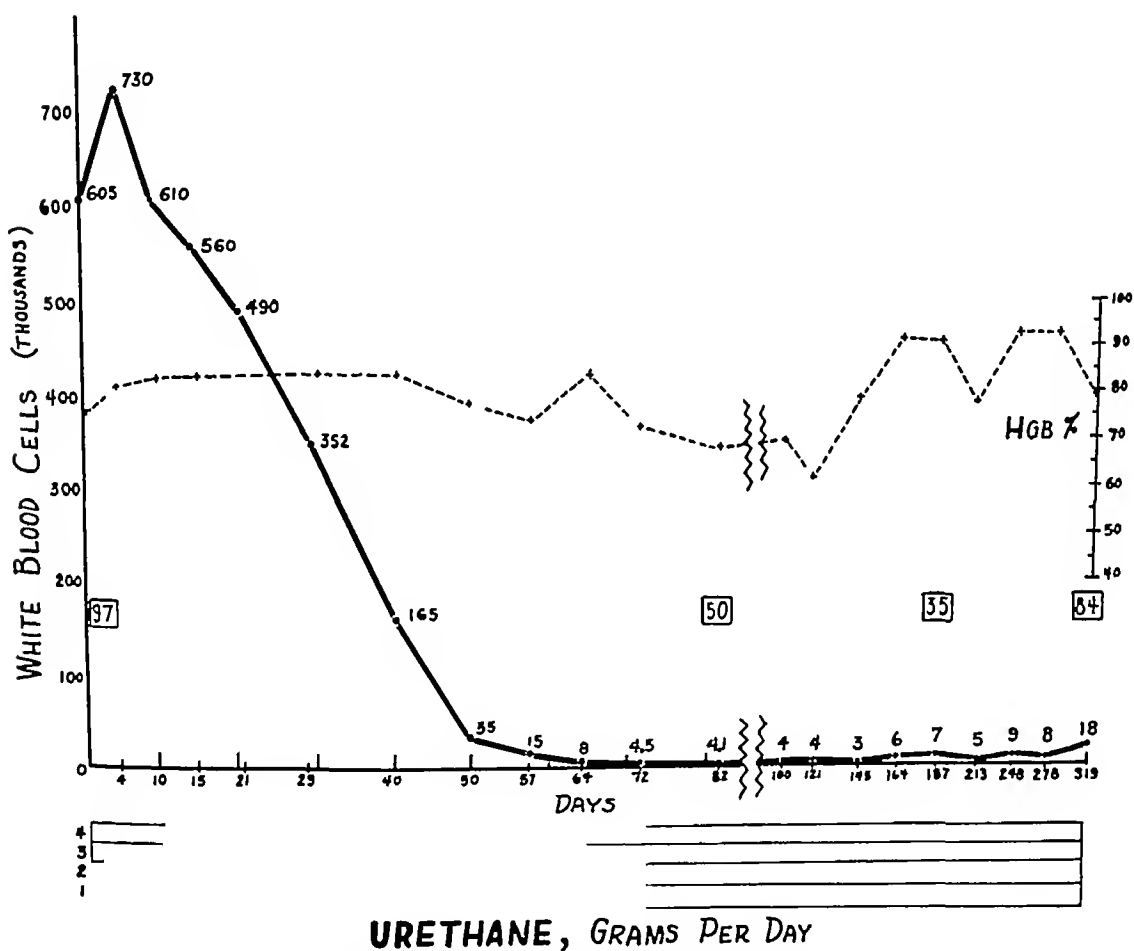


FIG. 2. CASE 2, CHRONIC LYMPHATIC LEUKEMIA IN A WHITE FEMALE, AGE 57  
(□ = PER CENT LYMPHOCYTES)

27, 1946, in a dosage of 2 Gm daily, in solution orally. Blood Hgb 14.5 Gm, WBC 58,000, lymphocytes 89 per cent. No effect of the drug could be detected after 299 days of treatment, (total dose 683 Gm), and it was stopped on May 15, 1947, when the blood count was Hgb 13.0 Gm, WBC 21,000, lymphocytes 93 per cent.

This case suggests that low-grade chronic lymphatic leukemia is resistant to urethane in a dosage of 2 Gm daily.

*Case 7* This white male, age 67, was hospitalized in March, 1944 for prostatic resection. At this time the blood picture of chronic lymphatic leukemia was found. He received a course of irradiation therapy in October, 1945. When first seen in the clinic on November 12, 1946, he was acutely ill, with findings of massive lymphadenopathy, splenomegaly, and marked pulmonary infiltration. Blood Hgb 7.5 Gm.

WBC 233,000, lymphocytes 99 per cent, Platelets 320,000 Urethane, 4 Gm daily, in solution orally, was started on November 16, 1946 On January 18, 1947 (sixty-second day of treatment), after a total dose of 225 Gm, the drug was stopped when the blood count was Hgb 6.9 Gm, WBC 5,600, lymphocytes 93 per cent The patient had become progressively worse He died in cardiac failure on January 31, 1947, despite apparent "improvement" in the abnormal lymphocytosis

*Case 8* This white male, age 61, had sudden severe pain in the left side of the abdomen in November, 1946 Examination disclosed generalized lymphadenopathy, marked splenomegaly, and marked lymphocytosis Blood Hgb 11.4 Gm, WBC 238,000, lymphocytes 98 per cent Urethane was started on November 9, 1946, 2 Gm daily, in solution orally Dosage was increased to 4 Gm daily on November 27,

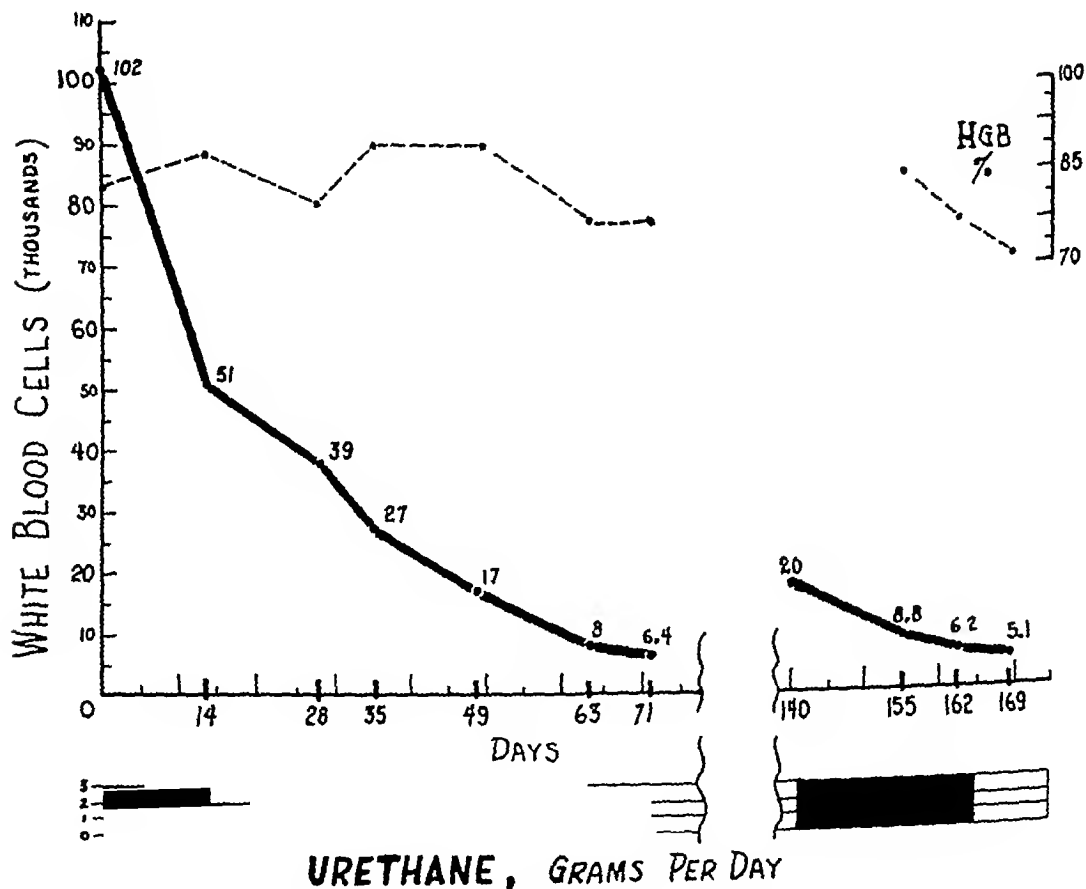


FIG 3 CASE 3, CHRONIC LYMPHATIC LEUKEMIA IN A WHITE FEMALE, AGE 54

1946, and to 6 Gm daily on December 5, 1946 Weakness, marked sweating, and insomnia developed On January 6, 1947, lymph nodes were estimated 80 per cent reduced in size but the size of the spleen was unchanged Blood Hgb 11.1 Gm, WBC 400,000 The dosage of urethane was decreased to 4 Gm daily until January 20, 1947 (sixty-third day of treatment) when it was discontinued (total dose 297 Gm) Blood Hgb 12.2 Gm, WBC 293,000, lymphocytes 97 per cent X-ray therapy was instituted with prompt fall in the leukocyte count, but little change in size of lymph nodes or spleen When last examined on August 25, 1947, moderate splenomegaly and lymph node enlargement were found His blood count showed Hgb 33.6 Gm, WBC 38,000, lymphocytes 93 per cent

This case is judged a urethane failure Although regression in lymph node size occurred there was no improvement in the splenomegaly or in the blood picture

*Case 13* This white male, age 52, presented generalized exfoliative dermatitis, lymphadenopathy, and lymphocytosis, diagnosed as leukemia cutis, of six months duration Urethane, 4 Gm daily, in solution

orally, was started on January 7, 1947 Blood Hgb 13.0 Gm, WBC 44,000, lymphocytes 83 per cent. No change in the patient's clinical condition was apparent when the drug was stopped 136 days later (total dose 521 Gm). Blood Hgb 11.5 Gm, WBC 21,000, lymphocytes 33 per cent.

TABLE 4—*Acute Leukemia*

Case	Original Status	WBC Start U	U (grams) U (days)	WBC End U	Result
#2 W 50 F	Untreated Monocytic	35,000	60 14	1,100	Good initial, but relapse 3 mo
#5 W 53 F	Untreated Myelogenous	79,000	26 10	8,000	Poor Died
#11 W 14 F	Untreated Myelogenous	240,000	64 20	250,000	Poor Died
#12 W 12 F	Untreated Aleukemic Lymph	1,800	14 7	900	Poor Died Aplastic mar- row
#14 W 42 M	Untreated Myelogenous	77,000	48 8	49,000	Poor Died
#15 W 15 M	Untreated Myelogenous	221,000	137 49	149,000	Poor Died 1 mo after treatment
#18 C 3 F	Untreated Myelogenous	56,000	26 13	300	Fair for 1 mo then re- lapse Marked decrease in size of kidney masses
#19 C 38 M	Untreated Myelogenous	110,000	67 17 (I V)	9,500	Left hospital No follow- up
#21 W 36 M	Untreated Lymphatic	200,000  39,000	28 14 (P O) 106 24 (I V)	39,000  132,000	Poor Died
#23 W 5 F	Untreated Myeloblastic	132,000	3 2 (I V)	2,700	Poor Died

*Case 16* This white male, age 45, noted enlargement in circumference of neck in December, 1946. When first seen on January 18, 1947, he presented generalized lymphadenopathy and moderate splenomegaly. Blood Hgb 13.99 Gm, WBC 265,000, lymphocytes 93 per cent, Platelets 112,000. Urethane, 3 Gm daily, orally, first in solution, then in capsules was started on January 27, 1947. The dose was soon increased to 4 Gm daily. By March 20, 1947 (fifty-first day of treatment), dangerous symptoms of drowsiness, weakness, anorexia, fever, petechiae, mucosal bleeding, and hematuria appeared. Blood Hgb 7.52 Gm, WBC 51,000, lymphocytes 93 per cent, Platelets 50,000. On April 1, 1947 (sixty-third day of treatment), after a total of 218 Gm, the drug was stopped. No significant changes in lymph node or splenic size were



demonstrable Multiple transfusions, followed by cautious x-ray irradiation produced slow improvement with cessation of hemorrhagic phenomena and marked reduction in the size of lymph nodes The blood picture improved, and when last seen on August 25, 1947, his blood count showed Hgb 13.20 Gm, WBC 81,000, lymphocytes 79 per cent, Platelets 64,000

This case is obviously another urethane failure It is possible that the drug produced critical hypoplasia of erythrocytic and megakaryocytic elements in the bone marrow

### *Acute Leukemias*

Table 4 summarizes the results in 10 cases of acute leukemia of various types In most cases, a prompt fall in the total leukocyte count followed urethane treatment Evanescent clinical improvement occurred in 4 patients (Cases 2, 15, 18, 19) Final information regarding 2 patients in this series is not available All other patients died during or soon after urethane therapy

### CASE REPORTS

*Case 2* This white female, age 40, had acute monocytic leukemia (Naegeli type) with fever, gingival necrosis, and extensive intracutaneous and mucosal bleeding of 2 weeks duration Urethane, 4 Gm daily, in solution orally, was started on August 21, 1946 Blood Hgb 7.2 Gm, WBC 35,000, immatures (promonocytes and monoblasts) 64 per cent A total of 60 Gm was given during the next ten days with rapid fall in leukocytes to 1,100, then to 650/cu mm Progressive anemia paralleled the fall in leukocytes On September 4, 1946, the blood count showed Hgb 1.38 Gm, WBC 1,300, immatures 26 per cent Despite these desperately low blood levels the patient felt improved, the gums healed and bleeding ceased There was gradual improvement and when the patient was sent home on October 4, 1946, the blood count was Hgb 4.95 Gm, WBC 2,400, immatures 36 per cent

It is noteworthy that sternal aspiration soon after termination of therapy showed striking reduction in numbers of blast cells as compared with the original aspiration

Continued improvement was maintained On December 4, 1946, the blood count showed Hgb 10.4 Gm, WBC 4,700, immatures none (commercial clinical laboratory) In mid-January, 1947, the patient fell, injured her mouth and apparently was precipitated into an acute relapse Urethane, 4 Gm, in solution orally, was resumed on January 23, 1947, when the blood count was Hgb 9.76 Gm, WBC 70,000, immatures 69 per cent The patient developed diffuse bronchopneumonia and died February 6, 1947 Blood Hgb 2.9 Gm, WBC 2,300

This was our first experience with the swift effect of urethane on the leukocyte count observed subsequently in most of our cases of acute leukemia The partial remission in this patient encouraged further trials in this form of leukemia

*Case 5* White female, age 53 Acute myelogenous leukemia Urethane, 2 to 4 Gm daily, in solution orally, was started on June 30, 1946 when the blood showed Hgb 12.4 Gm, WBC 79,000, immatures (promyelocytes and myeloblasts) 82 per cent Four days later the patient exhibited a phlebothrombosis of the right leg On July 9, 1946, urethane was discontinued (total dose 26 Gm) Blood Hgb 9.2 Gm, WBC 8,000 On July 23, 1946, the leukocyte count rose to 31,000 and urethane, 4 Gm daily, orally was resumed The patient died suddenly of pulmonary embolism, July 31, 1946, after a second course of 20 Gm of urethane when the blood count was Hgb 7.9 Gm, WBC 16,000

*Case 11* White female, age 14 This patient had acute myelogenous leukemia of two weeks duration, with generalized lymphadenopathy, hepato-splenomegaly, gingivitis, mucosal and intracutaneous bleeding On December 4, 1946, the blood count showed Hgb 12.2 Gm, WBC 360,000, blast cells 89 per cent A total dose of 68 Gm of urethane was given between December 30, 1946 and January 7, 1947, when the patient died after a progressively downhill course Blood Hgb 6.2 Gm, WBC 250,000

*Case 12* White female, age 12 This patient had acute aleukemic lymphatic leukemia with hemolytic staphylococcus aureus septicemia Urethane, 4 Gm daily, in solution orally was started on April 7, 1947

Blood Hgb 6.27 Gm, WBC 1,800, blast cells 82 per cent. Urethane was discontinued April 14, 1947 (total dose 14 Gm), when the blood count showed Hgb 6.6 Gm, WBC 900. Death occurred April 22, 1947, despite multiple transfusions and penicillin therapy.

Sections of marrow obtained at autopsy showed almost complete aplasia in contrast to the initial sternal marrow appearance of acute lymphoblastic leukemia.

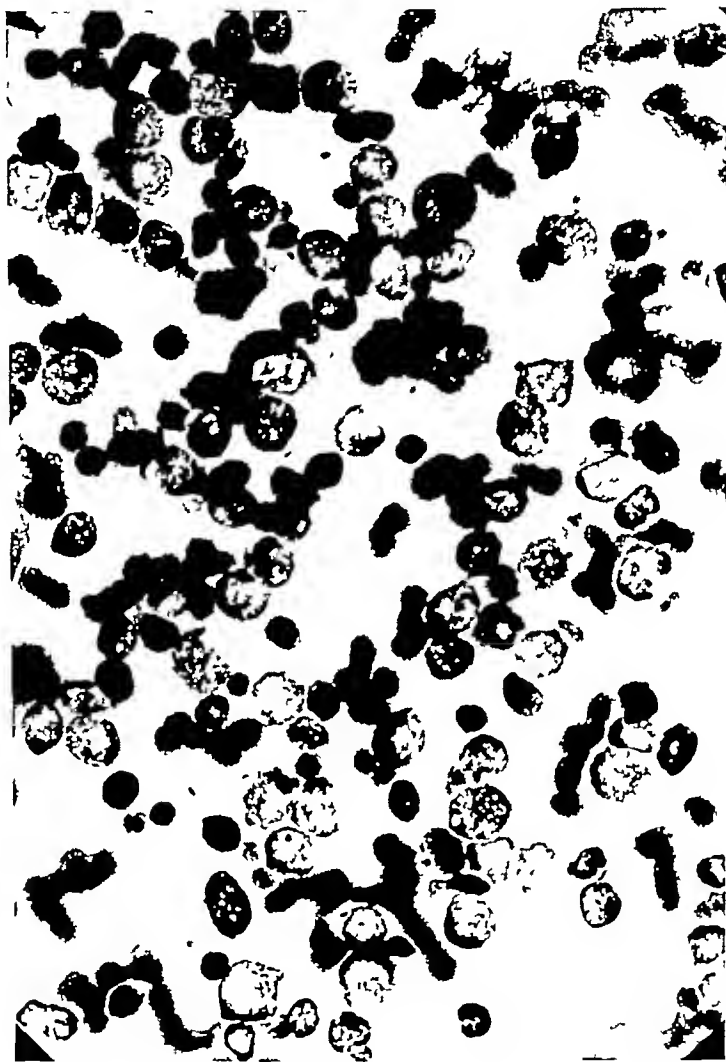


FIG. 4. BONE MARROW OF CASE 2 BEFORE TREATMENT.

*Case 14* White male, age 42. This patient had acute myelogenous leukemia and was moribund on admission. He was given 6 Gm urethane daily, in solution orally, for eight days without detectable effect clinically or hematologically. The patient died August 2, 1947 while still under therapy.

*Case 15* White male, age 15. Acute myelogenous leukemia. He had received 115 Gm urethane orally in 32 days, while in another hospital, with a fall in leukocytes from 221,000 to 19,300 but without clinical improvement.

Urethane, 3 to 4 Gm daily, orally in solution, was reinstituted March 1, 1947. Blood Hgb 13.8 Gm, WBC 74,000, immatures (blasts) 96 per cent. Because of severe nausea, vomiting, and diarrhea the drug was stopped March 8, 1947 (total dose 141 Gm). WBC 125,000. Irradiation therapy was started March 10, 1947 and given daily until discharged at his request on March 19, 1947, when his blood count showed Hgb 7.4 Gm, WBC 173,000. This patient died two weeks later.

*Case 18* \* Colored female, age 3 Acute myelogenous leukemia This patient had been ill for one month The outstanding physical finding was the presence of huge bilateral renal masses Urethane, 2 Gm , daily in solution orally, was started March 12, 1947, when the blood count was Hgb 4.3 Gm , WBC 41,000, immatures (blast cells) 40 per cent By March 19, 1947, the renal masses had decreased markedly in size (by palpation and urography) The drug was stopped on March 24, 1947, when the blood count showed Hgb 4.6 Gm , WBC 1,100, immatures none The patient seemed temporarily improved, but relapsed and died one month later

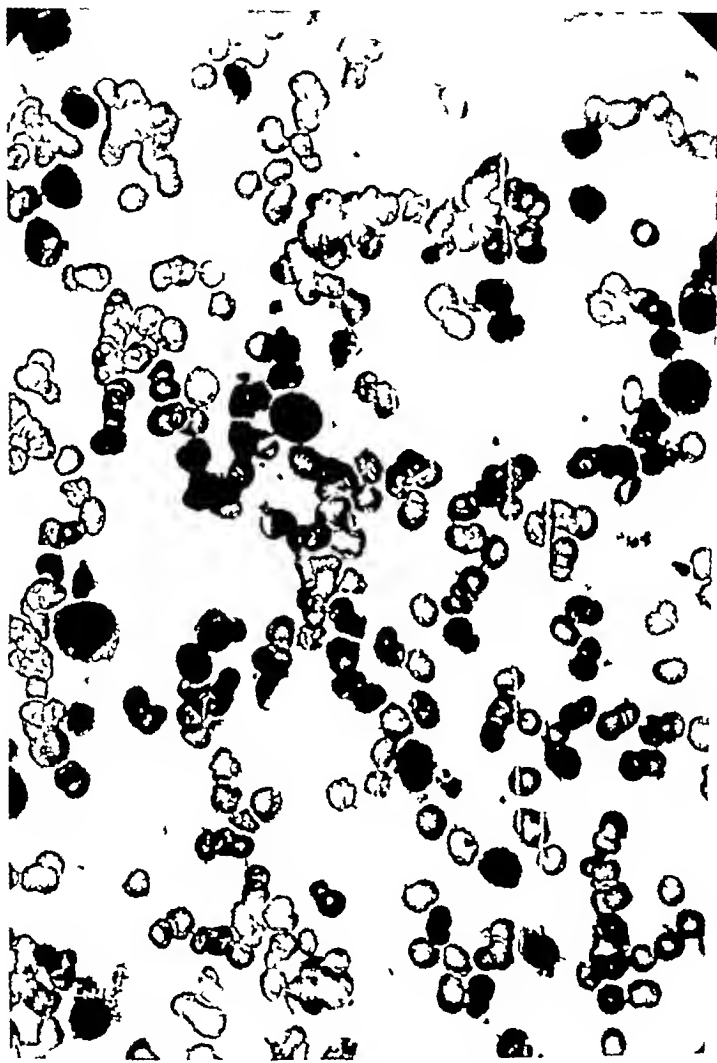


FIG 5 BONE MARROW OF CASE 2 AFTER TREATMENT

*Case 19* Colored male, age 38 Acute myelogenous leukemia Urethane, 3 Gm intravenously was started on May 9, 1947, and increased to 4 Gm daily the following day Blood Hgb 2.11 Gm , WBC 110,000, immatures (promyelocytes and myeloblasts) 90 per cent On May 25, 1947, after a total of 67 Gm intravenously, the patient showed improvement in strength and a 50 per cent decrease in the size of the lymph nodes The blood count at this time showed Hgb 5.9 Gm , WBC 9,500, immatures 73 per cent After receiving 2,000 cc of whole blood the patient was discharged June 10, 1947 when the blood count showed Hgb 7.8 Gm , WBC 5,500, immatures 69 per cent It has been impossible to obtain further information regarding this patient

\* This case observed at Philadelphia General Hospital, courtesy of Dr Paul Gyorgi

*Case 21* White male, age 30 Acute lymphatic leukemia This patient had been given 28 Gm of urethane in fourteen days, elsewhere, with fall in leukocyte count from 200,000 to 39,000. He was admitted to the Hospital of The University of Pennsylvania on May 26, 1947, in poor condition, and given preliminary dose of 3 Gm urethane, intravenously at which time the blood count was Hgb 8.8 Gm, WBC 21,000, immatures (prolymphocytes and lymphoblasts) 55 per cent. The dose was changed to 4 Gm daily on May 27, 1947, and again to 6 Gm daily on June 13, 1947. Blood Hgb 6.9 Gm, WBC 109,000, immatures 99 per cent. A total of 106 Gm urethane, intravenously, had been given over a twenty-four day

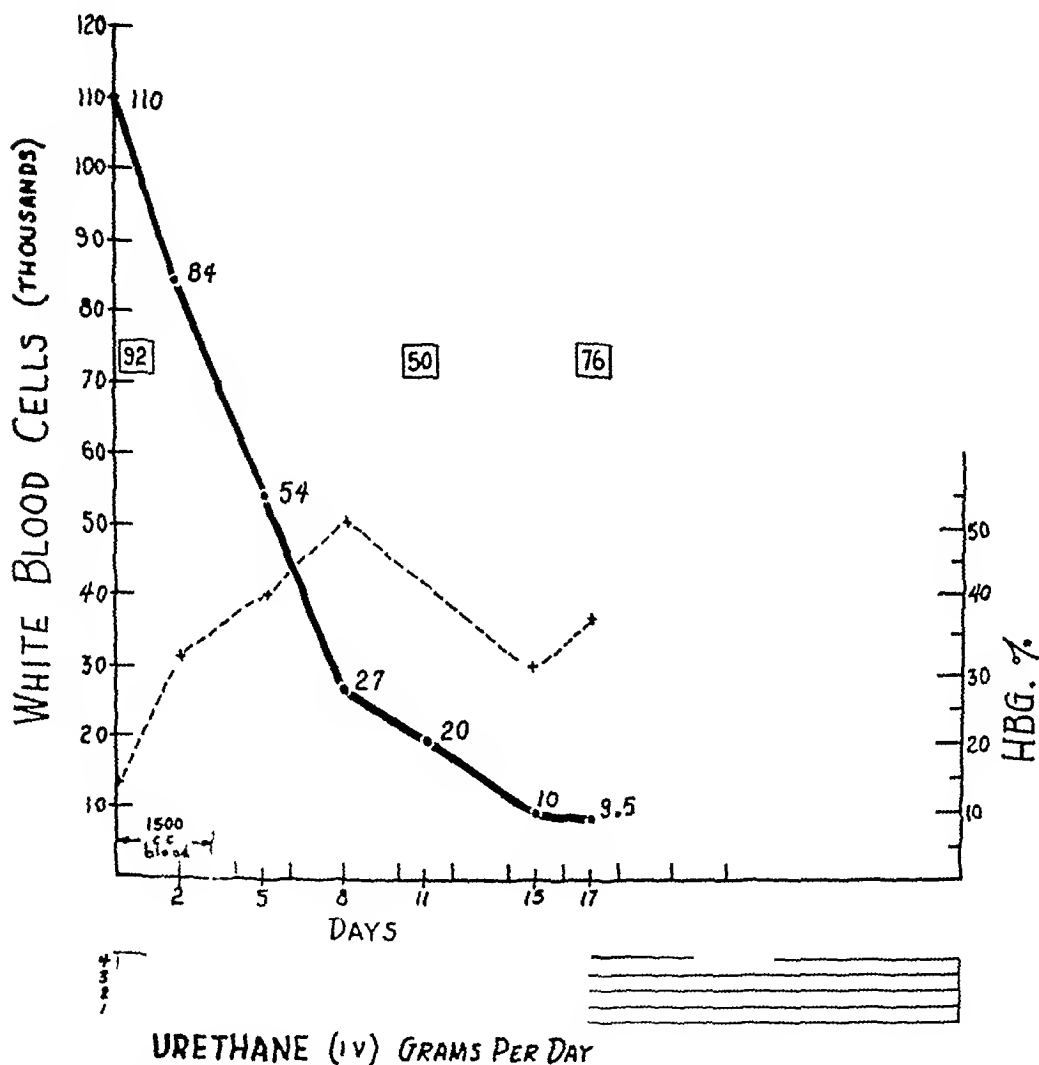


FIG. 6 CASE 19, ACUTE MYELOGENOUS LEUKEMIA IN A NEGRO MALE (□ = PER CENT BLASTS AND PROMYELOCYTES)

period when this patient died June 20, 1947. The blood count then was Hgb 8.6 Gm, WBC 132,000. The patient had also received 5,000 cc of whole blood during his hospital stay.

*Case 23* White female, age 5 Acute myelogenous leukemia One Gm urethane, intravenously, was given June 8, 1947, when the blood count was Hgb 7.02 Gm, WBC 132,000, blasts 90 per cent. On June 9, 1947, 2 Gm urethane was given intravenously. This was followed by a sudden rise in temperature to 105.2 F, rectally. Urethane therapy was discontinued. On June 12, 1947, marked diminution in splenic size was noted, and the blood count was Hgb 6.13 Gm, WBC 2,700. The patient died June 17, 1947, with a Hgb of 4.75 Gm, and a WBC of 750 cu mm.

### *Summary of Results*

It is apparent from the data presented above that satisfactory remissions as a result of urethane therapy occurred in 4 cases (1, 3, 4, 24) of our combined number of 24 cases. It is worthy of emphasis that of these 24 cases, at least 14 (Cases 9, 10, 17, 20, and all acute leukemias) and possibly 2 others (Cases 7 and 21) would, in our opinion, have proved hopelessly refractory to any known method of treatment of leukemia. It may be significant also that although urethane was withdrawn in Cases 8 and 13 because of poor response, these patients subsequently responded poorly to irradiation and radioactive phosphorous, respectively.

If the experimentally treated 4 terminal cases of chronic myelogenous leukemia are omitted from consideration, it is apparent that urethane produced a satisfactory result in 2 of the 3 remaining cases. Adequate response was obtained in 2 of 7 cases of chronic lymphatic leukemia. It becomes evident that of 10 "fair" cases of chronic leukemia, urethane was successfully used in 4.

### DRUG EFFECTS

*Toxicity* Nausea has been the predominant symptom of intolerance. Approximately 50 per cent of our patients exhibited mild or severe nausea commencing at any time during the course of oral administration of the drug. In a few cases, nausea was sufficiently severe to warrant interruption or cessation of treatment. Lesser toxic manifestations have been vomiting, anorexia, excessive sweating, diarrhea, and drowsiness.

The most dangerous toxic manifestation has been the appearance of evidence suggesting depression of all marrow elements (Cases 12, 16). Whether or not urethane produced or contributed to this effect, it is important to be wary of abrupt falls in erythrocyte or leukocyte levels during the period of urethane administration. It is significant that 2 patients in Paterson's series exhibited similar hypoplastic changes. Particular caution is advisable when leukopenic leukemia is under treatment, on the basis of Case 12. Rapidly developing anemia, in our experience, calls for immediate cessation of urethane therapy.

Urethane administered intravenously has produced only minimal side effects, such as transitory drowsiness, and transitory elevation of temperature. No local irritative phenomena have been observed. Particularly gratifying has been the absence of nausea, suggesting that the occurrence of this symptom after oral administration is due to gastric irritation. It is possible that enteric coated capsules or tablets may circumvent such nausea, and trials with such preparations are in progress.

*Rapidity of effect* The time required for a definite reduction in the leukocyte count (to 15-20,000) in favorable chronic cases averaged forty-eight days when the drug was given by mouth. In the acute leukemias, the effect was much more rapid, averaging ten days. It is believed that intravenous administration of urethane will produce effects more quickly than the above mentioned periods, although our data are as yet insufficient to establish this point.

*Mode of action* The manner and site of action of urethane are entirely unknown.

Haddow and Sexton<sup>1</sup> offer speculation regarding the possible action of urethane in remedying some deficiency in the process of leukocytic maturation in leukemia

*Basal metabolism and blood chemistry* In a small number of patients with chronic forms of leukemia, we observed that the basal metabolic rate fell in proportion to the decline in the total white cell count during urethane therapy

Marked reduction in elevated levels of blood uric acid was also noted in a few of our cases during therapy Case 19 had a blood uric acid of 11.3 mg per 100 ml on admission, which fell to 3.8 mg per 100 ml at the end of urethane therapy when the white blood count was 8,000 Case 4 had a reduction of blood uric acid from 8.9 mg per 100 ml to 4.2 mg per 100 ml while under urethane therapy

Blood urea nitrogen determinations were made before, during, and after urethane administration in 10 of our cases Elevation of the level of blood urea nitrogen occurred in no case during full urethane dosage

Blood levels of urethane have not been obtained A method of estimation has recently been published by Archer et al.<sup>3</sup>

### DISCUSSION

Urethane adequately administered in cases of chronic leukemia frequently produces a marked reduction in the total leukocyte count (83.3 per cent of our series) In fully responsive, favorable cases definite clinical improvement gradually follows Splenomegaly is reduced, enlarged lymph nodes become smaller, and a feeling of betterment is expressed The hemogram assumes a more normal appearance in its entirety Unfortunately, in a larger number of cases the fall in the leukocyte count is a spurious improvement In such cases, visceral pathology and anemia may fail to improve or may grow worse, and the patient may exhibit progressive deterioration Continued administration of the drug in such instances may lead to rapid clinical deterioration It is generally advisable to suspend the drug if clinical improvement fails to appear within one week after a satisfactory rate of fall of the leukocyte count has appeared It is furthermore probably advisable to discontinue urethane if no significant fall in the white cell count has occurred within sixty days from the start of treatment

It is impossible, from our small series of cases, to offer definite criteria for selection of patients for urethane treatment It seems likely (in agreement with Paterson et al.<sup>2</sup>) that chronic myelogenous leukemia responds better to urethane than does the lymphatic variety The drug has proved of little value in the acute leukemias, although further trials are justifiable in view of the swiftly fatal outcome of this form of leukemia

No information is as yet available as to the possibility of inducing repeated remissions in chronic leukemia with urethane, and if so, whether these can then be followed by successful repetitions of x-ray therapy If this should prove the case, then prolongation of the leukemic life-span as well as amelioration of symptoms may be possible In this regard it may be mentioned that in Cases 3 and 24, urethane successfully induced remissions after relapse from previously successful x-ray therapy Conversely, in 2 other cases (8 and 16), x-ray therapy successfully followed urethane failures This is in accord with the observation of the British

workers that urethane and irradiation therapy are not mutually exclusive in effect. Our evidence does indicate strongly that urethane is without clinical effect in terminal, long standing, repeatedly irradiated cases of chronic leukemia.

It is our impression that urethane will prove to be an occasionally useful adjunct in the management of chronic leukemia. The low cost and convenience of administration, in comparison with irradiation therapy are strong points in favor of this drug.

It is the opinion of the authors that in the treatment of leukemia in general, urethane is less consistent in effect than standard methods of irradiation therapy, and more consistent and more efficient than such modalities as Fowler's solution, benzol, and colchicine.

Our results, while not as striking, generally confirm the findings reported by Paterson and her co-workers. Urethane deserves further trial in the treatment of leukemias of all types.

#### SUMMARY

- 1 The results of urethane therapy in 24 cases of leukemia are described.
- 2 The average daily dose is 4 Gm orally or intravenously.
- 3 The drug is irregularly effective. Chronic myelogenous leukemia appears more responsive than the lymphatic variety.
- 4 Acute leukemias are not significantly altered in course by urethane.
- 5 Urethane produces a fall in the total leukocyte count in a majority of all types of leukemia. Clinical improvement does not necessarily follow.
- 6 Nausea is the most frequent side effect of urethane therapy. Possible marrow aplasia is the most dangerous toxic effect.
- 7 Urethane is of definite, but of limited value in the treatment of chronic leukemia. In some instances, it compares favorably with x-ray therapy, but in general, it is less dependable, particularly in its frequent failure to induce optimum return of normal red cell and platelet values, and optimum regression of organ infiltration.

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# THE EFFECT OF URETHANE ON THE NUCLEAR MORPHOLOGY OF CELLS OF THE GRANULOCYTE SERIES AS OBSERVED IN MARROW CULTURES AND LEUKEMIC BLOOD

By EDWIN E. OSGOOD, M.D., AND I. T. CHU, M.D.

INTEREST in urethane ( $\text{NH}_2 \text{CO OC}_2\text{H}_5$ ) and related carbamic acid esters in the treatment of metastatic malignant tumors and leukemias has been greatly stimulated by the report of Haddow and Sexton<sup>1</sup> on the effects of urethane on tumors in experimental animals, and of Paterson, Haddow, Thomas and Watkinson<sup>2</sup> on urethane therapy of human leukemias and malignant tumors. They demonstrated striking decreases in the leukocyte count and spleen size in certain patients with granulocytic or lymphocytic leukemias and in the size of metastatic nodules in a small proportion of patients with other malignant tumors. The observations of Paterson, Haddow, Thomas and Watkinson<sup>2</sup> have been confirmed by Goodman and Lewis<sup>3</sup> and by the authors at the University of Oregon Medical School.

Although urethane has long been in use as a narcotic and there is much literature on the action of the urethanes and related karyoklastic and karyokinetic poisons on the cells of lower forms of life,<sup>4</sup> we were unable to find any reference to morphologic changes produced by urethane in the nuclei of human cells.

The present study, using the technic of human marrow culture,<sup>5</sup> was undertaken as part of a long-term investigation of the factors influencing the fundamental phases of cell growth, namely, increase in size, mitotic and amitotic division, differentiation, and life span. This paper will be limited to the morphologic changes in cells of the granulocyte series produced by the action of urethane. Quantitative data on the comparative effects of urethane and methyl-bis (B-chloroethyl) amine hydrochloride obtained in the course of this study will appear later.

## METHOD

Marrow cultures were set up as previously described,<sup>6</sup> using sterile ascitic fluid as a source of protein instead of human cord serum. A marrow culture was prepared from each of 8 patients with miscellaneous diseases displaying essentially normal marrow pictures. In addition, the bloods of 4 patients with chronic granulocytic leukemia, 2 with acute lymphocytic leukemia, 1 with acute monocytic leukemia, and 1 with multiple myeloma with a plasmacytic-leukemia blood picture were cultured in the same way as marrow. Each culture was first thoroughly shaken in one vial and then equal parts were transferred to a series of vaccine vials, one of which was left as a control to which the solvent only was added and to the others equal volumes of varying concentrations of urethane or of methyl-bis (B-chloroethyl) amine hydrochloride in isotonic saline were added. Since no references were found giving the actual blood levels of urethane obtained in clinical therapy, a wide range of final concentrations was used, including 1:200, 1:1,000, 1:2,500, 1:5,000, 1:10,000, 1:20,000, and 1:40,000, although not all concentrations were used in each experiment. The morphologic data were based on the study of Wright's stained smears made at the same time from the control, urethane and nitrogen mustard cultures. The samples for the smears were removed three hours after the drugs were added and at approximately twenty-four hours.

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From the Division of Experimental Medicine, University of Oregon Medical School. Aided by a grant from the Medical Research Foundation of Oregon.



intervals thereafter for five days. The criteria of cell identification and classification were those given and illustrated by Osgood and Ashworth.<sup>7</sup>

### OBSERVATIONS

The morphologic changes in structure of the cells of the granulocyte series noted in the cultures containing urethane are illustrated in figure 1 and outlined in table 1.

The first change to appear was a 5 to 10-fold increase in the number of progranulocytes in process of mitotic division as compared to the control. This was noted as early as three hours and in concentrations as low as 1:40,000. The increase in mitoses was most marked at twenty-four hours and persisted for seventy-two hours. All phases of mitotic division were seen but most were normal appearing metaphases similar to those illustrated in figure 2 by Osgood.<sup>8</sup>

The most commonly observed change was the condensation of the basichromatin in the nucleus of the progranulocytes and granulocytes into dense blocks separated by clear spaces and still surrounded by a nuclear membrane as shown in figure 1-a. This appeared as early as three hours, steadily increased as long as the cultures were observed, and was most marked in the higher concentrations, although it was noted with all concentrations.

The alteration in morphology shown in figure 1-b was frequently observed in the progranulocyte and granulocyte stage. There seemed to be a loss of the nuclear membrane and imperfectly square or rectangular projections from a mass of clumped chromatin. A somewhat similar picture is seen in the anaphase of normal mitotic division when the cell is so oriented that the plane of cell division is parallel to the plane of the slide, but the number of these cells was greater than would be expected from the number of cells seen in the metaphase of division and it seems probable that at least some of the cells showing this appearance represent an intermediate stage of karyorrhexis between figure 1-a and figure 1-c.

The most striking but least frequently observed change is shown in figure 1-c. It consisted of the appearance within the cell of numerous fragments of densely-staining, structureless chromatin in round, ovoid or rectangular blocks with no nuclear membrane. This appearance was somewhat suggestive of the colchicine arrest of mitosis in the metaphase,<sup>9</sup> illustrated in figure 1 in Osgood,<sup>10</sup> but careful studies showed the points of difference outlined in table 2. It seems more probable, therefore, that the urethane effect illustrated in figure 1-c represents karyorrhexis or fragmentation of a nucleus similar to that in figure 1-a.

Double nuclei with no suggestion of fission of the cytoplasm were frequent in the progranulocyte and all subsequent stages of the granulocyte series in the urethane-containing cultures, yet the cells did not appear to be appreciably larger than the corresponding cell type with a single nucleus. They seemed to be most abundant in the granulocyte stage but appeared first in the progranulocyte stage as illustrated in figure 1-d.

Another change in morphology which is not illustrated was a marked decrease in size of some of the neutrophil lobocytes and rhabdocytes, both as compared to the control culture and to the average normal size of these cells. This change was even more noticeable in the blood of some of the patients treated with urethane over long periods of time.

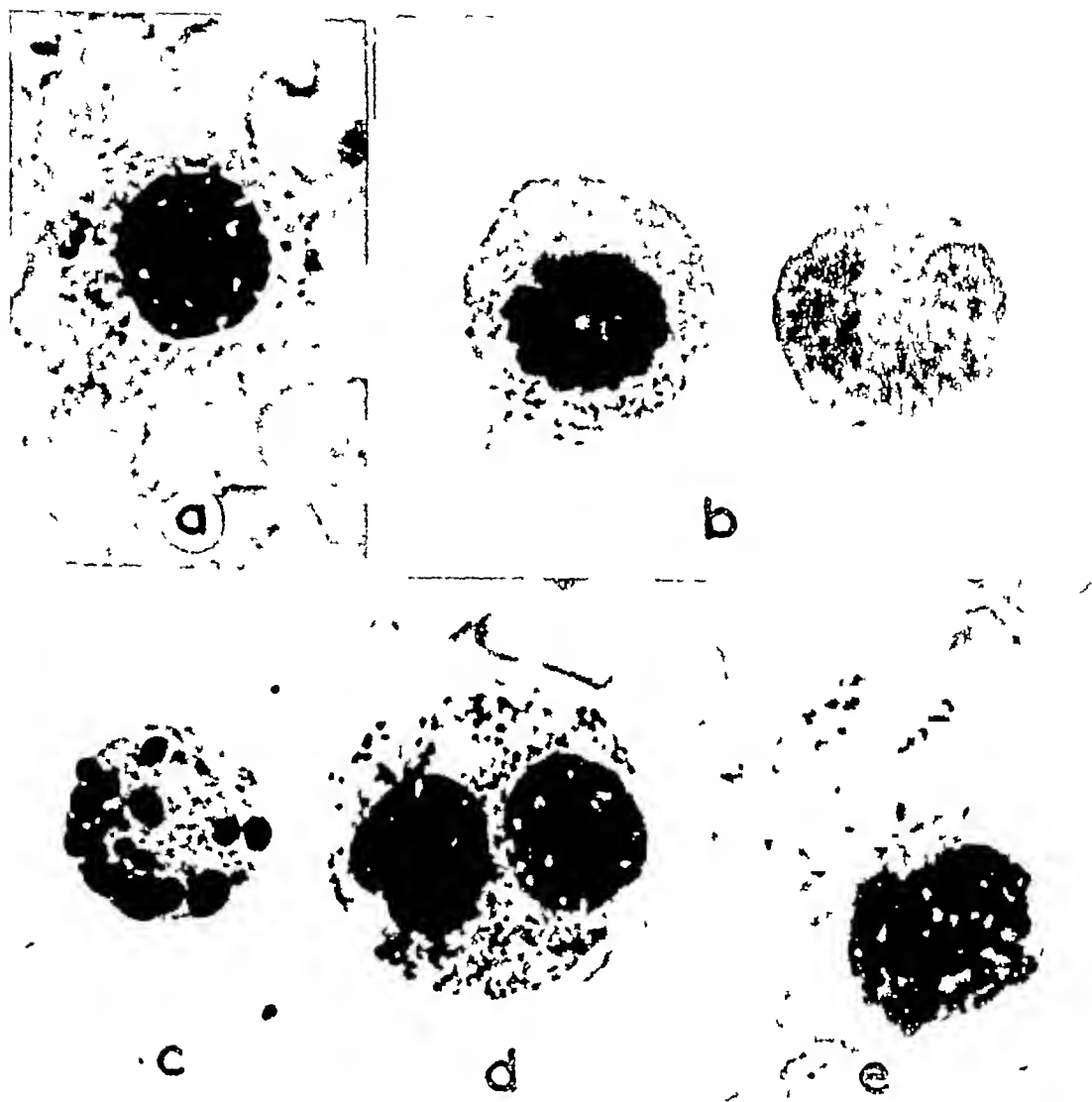


FIG 1 Photomicrographs of cells of the granulocyte series from urethane-containing culture (a-d), and leukemic blood (e), Wright's stain  $\times 1800$

a Neutrophil granulocyte showing chromatin clumps within the nucleus

b On the left is a cell which is either a neutrophil progranulocyte S in the anaphase of mitotic division viewed end-on or a neutrophil granulocyte showing an intermediate stage of karyorrhexis of the nucleus between figure 1-a and 1-c. On the right is a neutrophil rhabdocyte of perfectly normal morphology

c Neutrophil granulocytes showing fragmentation or karyorrhexis of the nucleus into numerous deeply-staining round or ovoid blocks of varying size

d Progranulocyte A with double nucleus

e This is a progranulocyte A from the blood of a patient with chronic granulocytic leukemia under treatment with urethane in process of amitotic division showing the mode of formation of the double nuclei. Note the rectangular projections from the nucleus with rounded corners. Amitotic division and double nuclei are almost never noted in the granulocyte series in either normal blood or marrow or blood or marrow from patients with leukemia who are not receiving urethane. Note that there is no tendency toward fission of the cytoplasm.

In addition, many of the cells of the granulocyte series developed dense, pyknotic, structureless nuclei and showed signs of loss of the cell membrane and disintegration.

None of these changes was noted in significant numbers in cells of the lymphocyte, monocyte, or plasmacyte series cultured or in the granulocyte series in the control and nitrogen mustard cultures. They have rarely if ever been observed by the authors in the course of an extensive study of the blood and marrow of patients with granulocytic leukemia or other diseases when urethane was not being given.

In each of the cultures and with each of the concentrations of urethane employed, many of the cells at each stage of development showed none of these changes and

TABLE 1 — *Approximate Percentage of Progranulocytes and Granulocytes Showing Morphologic Changes*

Changes observed	Control cultures 1 to 5 days	Cultures containing urethane	
		1 day	5 days
Normal	Over 99%	90%	Less than 20%
Mitoses	Less than 1%	7%	Less than 1%
Chromatin blocks (Figure 1-a)	Not seen	Less than 1%	60-70%
Separate chromatin blocks (Figure 1-c)	Not seen	Not seen	Less than 1%
Double nuclei (Figure 1-d)	Not seen or less than 0.1%	Less than 1%	15%

TABLE 2 — *Effects on Morphology of Cells Produced by Colchicine and Urethane*

	Colchicine	Urethane
Chromatin blocks	rectangular or square uniform in size number = or > normal no. of chromosomes	round, ovoid or with rounded corners unequal in size number < normal no. of chromosomes
Mitosis	arrested in metaphase	initial stimulation goes on to completion
Amitosis	not affected	greatly increased
Differentiation	prevented in higher concentration (1:100,000)	markedly abnormal but occurs in 1:200
Cell size	giant forms common	dwarf forms common
Nuclei	enlarged	pyknotic

appeared indistinguishable in morphology from cells of the corresponding stage of development in the control cultures or in the original marrow or blood of the patient. Such a normal appearing cell is illustrated by the neutrophil rhabdocyte in figure 1-b.

All of the changes noted in the cultures were also noted in blood or marrow of patients receiving 1 gram of urethane three times daily. The cell in figure 1-e is from the blood of a patient receiving urethane therapy. It shows the formation of double nuclei, apparently by amitotic division.

## COMMENT

The changes in morphology of the cells of the granulocyte series observed in blood and marrow cultures containing urethane suggest an effect of this drug on the state of aggregation or organization of the nucleoprotein within the cell nucleus, a tendency to disrupt the nuclear membrane and to interfere markedly with the normal process of cell division and cell growth. They resemble in many respects the changes described by Dustin,<sup>4</sup> Burt,<sup>11</sup> Piton,<sup>12</sup> and Chodkowski<sup>13</sup> and others whose work is cited in the references herein given,<sup>1,2,4,11-13</sup> as produced by a wide variety of unrelated "karyoklastic poisons," including many urethanes, many narcotics and arsenical compounds which seem to act by altering the permeability and integrity of cell membranes and nuclear membranes and the state of colloid aggregation of the nuclear and cytoplasmic proteins.

The morphologic effects produced by urethane were not seen in marrow cultures exposed to nitrogen mustard. While some of the changes superficially resemble those produced by colchicine,<sup>5, 9-10</sup> there are important differences. Colchicine, in adequate concentration, seems to arrest mitotic division completely, whereas urethane apparently stimulates division at first and the majority of cells complete division and differentiation.

In previous studies, using the marrow culture technic, of the action of ionizing radiation including 200 KV and 1,000 KV roentgen rays, neutron rays and radioactive phosphorus, it was shown<sup>5, 9, 14-16</sup> that in the dosage employed in treatment of leukemias, each modality of ionizing radiation inhibited the onset of the next division, mitotic or amitotic, rather than actually killing cells. In none of these experiments were morphologic changes similar to those noted in the urethane cultures observed, nor was the initial increase in mitoses and in total cell count which occurred in the urethane cultures noted.

We have confirmed the clinical observations of Paterson and her co-workers<sup>2</sup> that there is a great difference in clinical response of patients with apparently identical forms of leukemia to similar doses of urethane. It so happened that bloods of 2 of these patients were selected for culture before urethane was administered. Both patients were middle-aged women with long-standing chronic granulocytic leukemia with typical blood pictures. Both had been treated until resistant with local x-ray and not with the preferred total body irradiation at regular intervals. Both had refused further x-ray treatment. Case 1, unit number 157378, who responded well to urethane, had had a splenectomy several years previously because of the huge size of the spleen. Case 2, unit number 157231, which failed to respond to urethane, had a huge spleen reaching to the left iliac fossa 25 cm below the costal margin and extending across the midline. In other respects, they were as nearly alike as any two cases of chronic granulocytic leukemia could be. Both were given 1 gram of urethane three times a day.

In case 1, the leukocyte count had dropped from 33,800 to 7,700 after thirteen days of therapy, totalling 36 grams, and to 2,000 by the eighteenth day of therapy, totalling 51 grams, at which time the therapy was discontinued. In the course of the next twelve days, the leukocyte count dropped to 600 and, although the urethane had been stopped, it continued at this low level for many weeks, during

which time stomatitis from the agranulocytosis was controlled with difficulty by penicillin therapy. She then had a gradual reversion to a more normal leukocyte count.

In case 2, the initial leukocyte count before therapy was oscillating between 50,000 and 90,000 and was still about 50,000 after 141 grams of urethane were given in the course of thirty-nine days. The differential cell count pattern was not significantly altered and the size of the spleen was unchanged. Both patients had a good deal of nausea and some vomiting from the drug, and it is possible that in case 2 some of the drug was lost in the vomitus or was not taken, so during the last of the course the dose was increased to 4 grams daily, still without effect. This patient subsequently showed an excellent response to intravenous radioactive phosphorus therapy as far as leukocyte count and alteration in spleen size were concerned, although requiring somewhat higher dosage than the average patient who had not had x-ray therapy previously.

In the cultures of these two bloods to which urethane was added, however, the morphologic changes observed were, within the experimental limits of the method, indistinguishable in character and percentage of cells involved, suggesting that the clinical differences in response were due to differences in concentration of the active compound actually reaching the cell.

#### SUMMARY

In cultures by the marrow culture technic of human marrow and leukemic blood containing concentrations of urethane from 1:200 to 1:40,000, marked changes in the morphology of the cells of the granulocyte series were noted.

These changes were not noted in the control nor in duplicate cultures containing the methyl-bis (B-chloroethyl) amine hydrochloride form of nitrogen mustard in concentrations from 1:500,000 to 1:40,000,000, nor were they noted in previous studies of cultures containing colchicine or exposed to 200 kilovolt or million volt x-rays, neutron rays or radioactive phosphorus, nor in the bloods or marrows of patients with untreated chronic granulocytic leukemia, of healthy individuals or of persons with miscellaneous diseases.

The changes consisted of an early increase in number of normal mitoses in the progranulocytes, a steadily rising percentage of granulocytes and progranulocytes showing condensation of the chromatin in the nucleus into dense fragments separated by clear spaces, a progressive increase in the number of cells of the granulocyte series with double nuclei, affecting all cells from the progranulocyte stage, neutrophil lobocytes but appearing to be most numerous in the granulocyte stage, and the appearance in the cultures by 4 to 5 days of cells containing separated fragments of structureless material staining like basichromatin, which probably represents a karyorrhexis of the nucleus.

*Note.* Nothing in this article is to be construed as a recommendation of urethane for the clinical treatment of leukemias. While many years must elapse before its place in therapy can be evaluated, it does seem worthwhile to give urethane a trial for metastatic malignant tumors. Our present impression is that either radioactive

phosphorus or total body irradiation with x-rays given in small regularly spaced doses is far superior to urethane in the treatment of leukemias<sup>17</sup> When the cells become resistant to radiation therapy, urethane may be worthy of a trial

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## ANTIBIOTICS IN CHRONIC LEUKEMIA

By DUDLEY MERRILL, M D

THE day by day care of patients with chronic leukemia of any type is one which will tax the ingenuity of the physician to the utmost. These patients can sometimes be carried along for several, if not many, years, in comparative comfort with X-ray therapy and transfusions as often as necessary to maintain a hemoglobin between twelve and thirteen grams. One of the characteristics of these patients is their liability to infections and their extremely poor resistance to them. In fact, infection is one of the common causes of death in this particular group.

Prior to the discovery of the sulfonamides, penicillin, streptomycin and tyrothricin, an attack of pneumonia, a cellulitis of the throat, or an infected hematoma often resulted in death in a few days from overwhelming sepsis. This was true even in patients whose general condition was such prior to the infection that many weeks or months of active life could have been anticipated in spite of the leukemia.

For many years, it was seriously questioned by many authors whether X-ray therapy actually prolonged life in patients with chronic leukemia. It is practically impossible to prove this proposition statistically because of the extreme variation of life expectancy in these patients without any treatment whatever. When life expectancy ranges from six months to twelve years, an average or a mean becomes a mathematical figure of little or no significance when applied to an individual case.

The same is true of any attempt to evaluate in a statistical fashion the effect of antibiotics, nitrogen mustards, urethane or Fowler's solution on the course of leukemia.

The extraordinary value of the antibiotics in the management of infections in general has now been conclusively established. These drugs have also revolutionized many of our long accepted medical and surgical beliefs as well as many of our apparently well established rules of prognosis.

The care, as well as the prognosis, of patients with pneumonia, meningococcus meningitis, spreading streptococcal infection, osteomyelitis and bacterial endocarditis, have been profoundly altered in the past ten years.

One of the most revolutionary techniques for the handling of mild or severe cellulitis and lymphangitis localized or spreading, including even the face and nose, has been the injection of penicillin in large doses directly into and around the infected tissues. Rose and Hurwitz<sup>1</sup> have demonstrated the complete safety and great usefulness of this method. The superiority of this approach over parenterally administered penicillin at a distant site is not surprising when one considers the immense concentrations of penicillin achieved at the site where it is most needed. Comparable concentrations from penicillin injected at a distance from the infection, either intramuscularly or intravenously, obviously would necessitate massive doses. The simplicity and inexpensiveness of the procedure are also in its favor.

Luckily, none of the effectiveness of the antibiotics depends upon the integrity

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of the leukocytes. Thus, they are equally efficacious in coping with infection in patients with profound disorders of the white blood cells such as agranulocytosis, aplastic anemia, or any form of leukemia. Two cases\* are presented to illustrate

### CASE REPORTS

*Case No. 1 J. J.* This 15 year old boy was diagnosed as having leukemia after having been 'run down' for about four months. His blood count showed Hgb 34 per cent (S), RBC 169, and WBC 25,600. The smear showed 60 per cent large immature white cells. These were classified as either immature monocytes or histiocytes by a consulting hematologist. Treatment was decided upon in spite of the obviously poor prognosis. He was repeatedly transfused and given small doses of x-ray therapy with rapid symptomatic improvement.

About a month after treatment was started, he developed a painful hematoma in his right calf and smaller ones in the sole of his left foot and in his left cheek. In the next week his temperature rose gradually to 105 F in spite of full doses of sulfadiazine by mouth. The hematoma of his leg increased in size, induration, redness, and painfulness, until it occupied an area 15 x 10 cm. It finally broke down and discharged vast quantities of serosanguineous material. Coping with such a large ulcer which oozed such enormous quantities of serum, blood, and pus, and was extremely painful when dressings were changed, was finally solved by Doctor Robert Linton, who placed the leg in a cast and packed the ulcer with gauze impregnated with tyrothricin ointment. There was a dramatic improvement in the boy's general condition, with a drop of his temperature to normal. In three days' time, he was able to be up in a wheel chair. When the cast was changed after thirteen days, the ulcers were filling in rapidly. Eighteen days later, he was allowed home, where he was comfortable getting about in a wheel chair for the next month except for one brief return to the hospital for further transfusions. Finally, however, he had a recurrence of multiple hematomas, his white count rose to 190,000 with 98 per cent extremely immature cells, and he went downhill fairly rapidly in spite of x-ray therapy, transfusions, and tyrothricin applications to his ulcerated hematomas. He died approximately five months after the diagnosis was established and eight months after his first symptoms.

Postmortem examination established the diagnosis of monocytic leukemia.

*Case No. 2 Mrs. M. M.* This 74 year old woman was first seen because of anemia. She had a Hgb 46 per cent (S), RBC 190, WBC 3,250. The differential showed P 44, L 32, M 24, and 2 nucleated RBC/100 WBC. The platelets were normal, the RBC deeply stained, and there were many tailed cells. It was felt that her blood picture was consistent with pernicious anemia.

After one month on liver extract, 15 units intramuscularly once a week, her Hgb was 35 per cent (S), RBC 155, and WBC 4,500.

A sternal biopsy was then done and reported by Doctor H. E. MacMahon as follows: 'The overall picture is one of aplastic anemia, but there are tiny foci showing a picture consistent with pernicious anemia under treatment.'

Large doses of folic acid, liver extract powder, and intramuscular liver extract were given, but there was no response whatever. The patient was kept in excellent health without any complaints for a whole year by repeated blood transfusions.

Then increasing numbers of abnormal white cells began to appear, coincident with the development of areas of cellulitis on both sides of her neck and in both nostrils. The next day, penicillin, 25,000 units every three hours intramuscularly, was started, but in two days' time the cellulitis had spread and now involved the lower lip and cheek which were enormously swollen. She was then given 50,000 units of penicillin in 5 cc of saline directly into the infected areas. There was rapid improvement with resolution of the areas of cellulitis without any breaking down of the tissues. Parenteral penicillin was stopped nine days after the local injection had been given.

Six days later, however, a new area of cellulitis on the left side of the lower lip had appeared and paren-

\* Both of these cases are reported through the courtesy of Dr. Arthur N. McKechnie of Cambridge, Mass.



teral penicillin every three hours was again started. Five days later, this was changed to single daily injections of 300,000 units of penicillin in peanut oil and beeswax because of breaking down and abscess formation in the areas previously injected with penicillin in saline.

On this treatment, the areas of cellulitis about the lips and mouth subsided but she developed many new areas about the chin, cheeks, neck, right upper arm, and both buttocks. Her white count rose to 64,600 with more than 50 per cent extremely immature forms.

All therapy was finally abandoned, and the patient died about ten weeks after the first appearance of the areas of cellulitis in her neck. She had received a total of 19,575 cc. of whole blood in the course of fourteen months, and for twelve of those months she had had extraordinarily few, if any complaints.

Autopsy showed monocytic leukemia and extensive hemosiderosis of liver, spleen, lymph nodes, and bone marrow, consistent with hemochromatosis. This latter finding was presumably due to her enormous number of transfusions.

### CONCLUSION

The new antibiotic drugs—sulfonamides, penicillin, streptomycin, and tyrothricin, have proved useful in the management of the infectious complications in leukemia and other disorders of the bone marrow.

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# FOLLICULAR LYMPHOBLASTOMA

## A REPORT OF SIX CASES

By OVID O MEYER, M D

FOLLICULAR lymphoblastoma has been a subject of considerable interest since 1925 when Brill, Baehr, and Rosenthal<sup>1</sup> reported the 2 cases which made the medical profession really cognizant of this entity. They called the condition generalized giant lymph follicle hyperplasia of lymph nodes and spleen. Much earlier, in 1901, Becker<sup>2</sup> had described a case which probably was this disease, and between 1912 and 1920 descriptions<sup>3-5</sup> of a few additional cases were reported. In their original report Brill, Baehr, and Rosenthal<sup>1</sup> concluded that the lymph node disease was probably benign, but in a subsequent report published two years later Baehr and Rosenthal<sup>6</sup> concluded on the basis of six cases studied, that the condition was malignant. In the same year, however, Symmers<sup>7</sup> described 3 cases on the basis of which he concluded that the condition was benign from the "standpoint of prognosis." In an extensive report published in 1938, Symmers<sup>8</sup> described, with the histologic picture, cases of giant follicle hyperplasia which later were transformed into polymorphous cell sarcoma of the lymph follicles, others which appeared to terminate as Hodgkin's disease, and still others which ended as "lymphatic" leukemia. He stated that giant follicular lymphadenopathy, with or without splenomegaly, was probably inflammatory or toxic in origin and usually amenable to mild roentgen therapy. In a communication published in 1946,<sup>9</sup> he stated that he had seen 5 histologically proved cases in which roentgen therapy was followed by apparent cures for periods of 3, 4½, 5, 9½, and 12 years. Another patient survived nine years without any treatment. Baggenstoss and Heck,<sup>10</sup> on the other hand, agree with the conclusion which Baehr, Klemperer, and Rosenthal<sup>11</sup> had arrived at by 1931, namely that the disease is a form of lymphosarcoma which in its early stages presents the histologic picture of follicular hyperplasia but is later characterized by a conglomeration of the follicles and diffuse infiltration of the lymph nodes by polymorphous lymphoblasts. It was at this time that the term "follicular lymphoblastoma" was proposed.<sup>11</sup> By 1940, Baggenstoss and Heck<sup>10</sup> had collected 59 cases from the literature and 13 of their own, a total of 72. In 1941, Gall, Morrison, and Scott<sup>12</sup> reviewed 63 cases from biopsy or necropsy material submitted to the laboratory of the Massachusetts General Hospital. Since publication of this report, more than 50 additional cases<sup>13-39</sup> have been recorded through 1946. These include 15 cases with skin manifestations reported by Combes and Bluefarb.<sup>30</sup> The present paper reports 6 additional cases.

The chief characteristics of follicular lymphoblastoma are outlined by Baehr, Klemperer, and Rosenthal.<sup>11</sup> Enlargement of the lymph nodes is due to enormously enlarged lymph follicles, a single one of which may fill a low power microscopic field. The follicles resemble huge germinal centers consisting of lymphoblasts with

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frequent mitotic figures. The periphery of each large follicle is surrounded by a narrow zone of small lymphocytes whose nuclei stain darker. The splenomegaly may be enormous as a result, chiefly, of enlargement of Malpighian bodies. There are no abnormal cells in the blood. Nor is anemia and cachexia present until the end stages of the disease. There is a tendency to lymphatic infiltration in the lacrimal gland, which gives rise to unilateral exophthalmus, and there is a tendency to involvement of serous membrane with pleural or peritoneal serous or even chylous effusion. The disease is remarkable for its chronicity and its extreme radiosensitivity. No neoplastic disease responds more promptly to relatively small doses of roentgen or radium therapy. Recurrences in widely separated parts of the body usually take place after varying intervals until eventually, often after many years, there is radioresistance.

The present report is of 6 cases seen at the State of Wisconsin General Hospital since 1941. Two, cases 2 and 6, were of particular interest because of bone involvement. Three of the patients were women, 3 were men. The ages were 54, 45, 44, 61, 82, and 60 respectively.

### CASE REPORTS

*Case 1.* L M M, age 54, a white farm housewife, was admitted September 3, 1941. She complained that for six months she had been afflicted with shortness of breath and a cough. In the spring of 1941, she noted a painless swelling of the right side of the neck. She saw a physician on July 3 and was hospitalized. A left pleural effusion was demonstrated, and thoracentesis was performed on three occasions, each of which was followed by temporary relief of dyspnea.

Examination showed an obese white woman, able to sit up in bed. The trachea was deviated to the right. There were 2 cm. nodes in the right cervical region, several 1 cm. nodes in the left posterior cervical chain, and a healed surgical scar of biopsy on the right. The nodes were freely movable, nontender, and without induration. No other enlargement of lymph nodes was demonstrated. There were signs of a massive fluid accumulation in the left thorax. The blood pressure was 170/90. The liver extended 3 cm., the spleen 8 cm. below the costal margin.

Laboratory studies showed a hemoglobin of 14.5 Gm. (90 per cent), erythrocytes 3,930,000, leukocytes 5,500, neutrophils 64, lymphocytes 32 per cent, monocytes 2, eosinophiles 2 per cent. Blood Wassermann was negative. A roentgenogram of the chest as read by Dr. L. W. Paul showed massive left-sided opacity, presumably due to pleural effusion. Bucky film after aspiration of fluid did not demonstrate enlargement of mediastinal nodes. The tuberculin test was negative.

Thoracentesis on three successive days resulted in the withdrawal of 1500 cc. of chylous fluid on each occasion. No organisms were found in the fluid, and it was negative for acid-fast bacilli. The specific gravity was 1.023, and albumen 2 per cent, there were many red blood cells. Biopsy of a node from the right cervical region on September 6, 1941, showed the findings of follicular lymphoblastoma.

The patient received eight treatments of 150-r to 200-r each in air (H V L = 1.05 mm. distance 50 cm.) between September 8 and 13, 1941, and she went home. In October she returned to the outpatient department. Earlier in the month she had required a thoracentesis. There had been no gain in weight, but she felt better. Her cough, however, and the signs of a small fluid accumulation in the left pleural cavity persisted. There were no palpable lymph nodes, the spleen was about 6 cm. below the costal margin. On January 8, 1942, the patient reported a ravenous appetite, an 8 pound gain in weight, and for three weeks recurrent backache in the mid-lumbar region which was severe enough to cause much loss of sleep. The cough had disappeared. Examination showed tiny cervical and axillary nodes. The spleen was 2-3 cm. below the costal margin. There was muscle spasm and tenderness in the right lumbar region. A roentgenogram of the lumbar spine was negative for bone or joint disease. Three treatments of 150-r each were given to the spine. Subsequently, after the patient had returned home, massage and diathermy were administered, since the pain persisted.

She was readmitted to the hospital on April 27, 1942, suffering torturing pain in the back and down into the legs. She had increasing difficulty in walking and loss of control of the bladder and bowels.

Physical examination at this time revealed signs of a moderate accumulation of fluid in the left pleural cavity, the spleen was just palpable, and there was no enlargement of the lymph nodes. The patient could no longer move the lower extremities, the deep reflexes had ceased, and the vibratory sense and position sense were absent. There appeared to be deformity of the 8th thoracic vertebra.

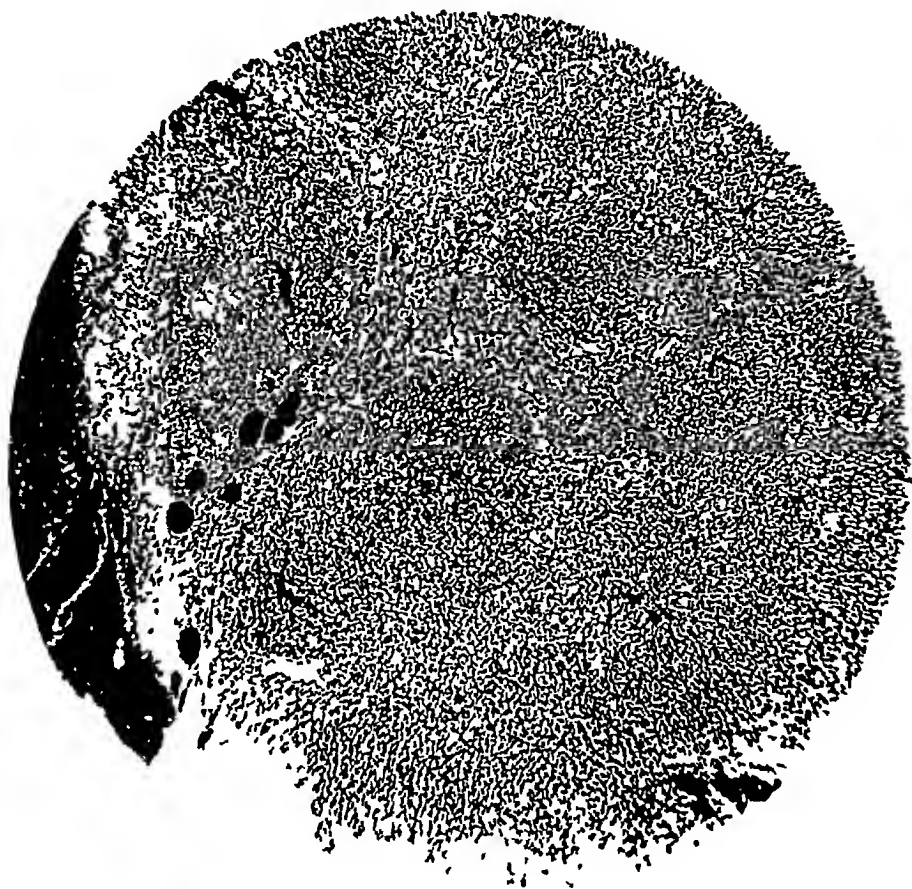


FIG. 1. SECTION OF A CERVICAL LYMPH NODE OF PATIENT L. M. M., CASE NO. 1.  
MAGNIFICATION 30X.

Laboratory studies showed the hemoglobin to be 11.4 Gm (70 per cent), leukocytes 4,500 with 76 per cent neutrophils. A roentgenogram of the dorsal and lumbar spine showed no definite bone lesions.

It was believed that the patient had a lesion compressing the cord, and five treatments totaling 1100-r in air (H.V.L. = 1.05 mm, distance 50 cm) were administered to the dorsal and lumbar regions. She returned to the hospital for the last time on July 9, 1942. Her condition has not improved, ten days before, her legs had become slightly flexed at the hips and knees and it was almost impossible to move them.

There was anesthesia in both legs, due to apparent sacral segment lesion, and in the right leg it extended to lumbar 4. Deep reflexes were absent. Roentgenograms of the dorsal and lumbar spine were negative. The laboratory studies were not significantly different from those made in April. Six treatments of 200-r each in air (H.V.L. = 2.4 mm, distance 60 cm) were administered to the dorsal and lumbar spine between July 1 and 7, 1942, and the patient was discharged. She died at home about two months later and autopsy was not obtained.

*Comment* Involvement of the pleura with effusion was prominent in this patient. The unproved but apparent involvement of the spinal cord was unusual. The course was rapid, death occurring within 18 months of onset of the lymph node enlargement.

*Case 2* C A J, age 45, an American housewife of Dutch descent, was admitted on May 13, 1946, complaining of pain in the left leg. Her illness had begun three years before with episodes of gaseous distension, nausea, and diarrhea. Several months later she noted enlargement of nodes in the inguinal regions, enlargement of the abdomen, and then the presence of lumps in the neck and axillae. Biopsy was made and x-ray therapy given. The nodes disappeared. A few months later she complained of pains in the arms, legs, and lower abdomen. These gradually decreased without specific therapy. After this she had been able to do hard work until two months before admission when she again suffered pain in the left leg and lower back.

Physical examination showed a well-developed, well-nourished, though pallid woman. The largest of a few palpable nodes, 2 cm in diameter, was in the right axilla. The liver and spleen were not palpable.

Laboratory studies showed 4 plus glucose in the urine, hemoglobin 11 Gm (70 per cent), erythrocytes 3,415,000, leukocytes 2,700 with neutrophils 69, eosinophiles 0.6, small lymphocytes 7, atypical lymphocytes 2.6, intermediate and large lymphocytes 3.4, young lymphocytes 5, monocytes 11.4, metamyelocytes 0.8, neutrophilic myelocytes 0.2 per cent. Reticulocytes numbered 1.4 per cent. The volume of the individual cell was 94.2 cu microns. A roentgenogram of the chest showed no enlargement of mediastinal nodes. The roentgenogram of the pelvis as read by Dr. L. W. Paul showed areas of increased density in the ischial rami, particularly on the left side, with a suggestion of a little mottling in the upper ends of the femurs. These changes were thought by Dr. Paul to be consistent with early involvement of these bones by a lymphoblastoma.

Biopsy of an axillary lymph node was performed, the description of Dr. Walter Jaeschke being as follows. This moderately enlarged node has a moist grey medullary appearance on cut section. Throughout, there are giant follicles separated by a lymphoid stroma. In the stroma, there are numerous eosinophiles. Reed-Sternberg cells are not identified. Diagnosis: giant follicle lymphoblastoma.

The patient received 600-r in air of high voltage roentgen therapy (H V L cu = 1.05 mm distance 50 cm) to the right and left axilla and a similar dosage to the anterior and posterior pelvis. She was discharged on May 22, 1946. Her local physician reports that the patient died in July, 1946.

*Comment* This patient, who had symptoms for three years before coming under our observation, had definite anemia by this time. There is in the literature little to suggest involvement of bone with follicular lymphoblastoma, but since it is common in other types of lymphoblastoma, its occurrence in 2 of the 6 cases here described is probably not surprising.

*Case 3* E R H, a 44-year-old white male, was first admitted to the Wisconsin General Hospital on April 28, 1942, complaining chiefly of lumps on the head and left side of the neck. He had had an enlarged node behind the right ear as long as he could remember. About nine months before admission, he had first noted three small lumps on the right side of the head. These slowly increased in size, and a week before admission another lump appeared on the left side of the head. A biopsy of a node from the scalp showed hyperplastic follicles. He had lost 22 pounds in weight.

Physical examination showed this patient to be a well-developed, well-nourished man of good color. On the right side of the head were three firm, but movable, masses measuring 2 x 3 to 2 x 4 cm. On the left side, also in the region of the scalp was a single smaller node, and in the left submaxillary region was a firm node measuring 3 x 5 cm. In the neck were several smaller nodes and there were also small nodes in the right postauricular, the axillary, epitrochlear, and inguinal regions. Blood pressure was 200 systolic, 120 diastolic. The liver extended 3 cm, the spleen 2 cm below the costal margin on deep inspiration.

Laboratory studies showed a hemoglobin of 15.9 Gm (100 per cent), erythrocytes 4,280,000, leukocytes 8,600 with neutrophils 76 per cent, lymphocytes 23 per cent, eosinophiles 1 per cent. Blood

Wassermann was negative. The basal metabolic rate was plus 37. Roentgenograms of the chest showed cardiac enlargement. X-ray of the skull was negative. Biopsy of the node in the left submandibular region was interpreted by Dr. W. D. Stovall as giant folliculoma (follicular lymphoblastoma).



FIG. 2. SECTION OF AN AXILLARY LYMPH NODE, CASE NO. 2. MAGNIFICATION 60X.

The patient was discharged on May 5, 1942, to report to the outpatient department for roentgen therapy. He has been seen repeatedly in the outpatient department and has twice been readmitted to the hospital, in May, 1943, and on January 11, 1945, at which time he complained of dyspnea, orthopnea, precordial pain, and cardiac arrhythmia. For five months he had been hoarse and for a week had noted enlargement of the spleen with pain in this region. The right side of the neck was swollen.

The patient was apprehensive. There was general superficial enlargement of the lymph nodes, several small nodules scattered in the subcutaneous tissue, signs of bilateral intrapleural fluid. The liver extended 6-8 cm below the costal margin, and the spleen was palpated 16 cm below the costal margin. The blood pressure was 185 systolic, 120 diastolic.

Laboratory studies included a urinalysis with 5 to 10 casts per low-power field, specific gravity 1.026. The blood count was still essentially normal. A roentgenogram of the abdomen showed enlargement of the spleen and liver. An x-ray of the chest showed the presence of fluid bilaterally. There was also moderate enlargement of the mediastinal nodes.

It was thought that much of the symptomatology and the pleural effusion were attributable to the cardiac disease rather than to the lymphoblastoma. However, the patient received 12 treatments of 150-r each in air (H V L cu = 1.05 mm distance 50 cm) distributed to the spleen, right cervical, and mediastinal regions. He was discharged January 25, 1945. On April 15 of that year, his physician reports the patient died of cardiovascular renal syndrome.

*Comment* The duration of this case of lymphoblastoma could not be ascertained but seemingly it was long if the original "lumps" are significant. If not, then the course was short, death resulting from the cardiac and renal failure induced by the severe and persistent hypertension. Pleural effusion is common in follicular lymphoblastoma, but here it was thought to be cardiac rather than lymphomatous in origin.

*Case 4* Mrs. J. H., age 61, the wife of a missionary to China for many years, visited the outpatient department on March 5, 1946, with the complaint of a lump in the right groin. Sixteen years before, while in China, her left eye had been removed for what was found to be a malignant tumor of unknown type. On July 24, 1941, 32 cm of rectum and sigmoid were removed and a colostomy performed for a neoplasm which was localized and found to be an adenocarcinoma. On May 17, 1943, a tumor in the left inguinal region was removed. A microscopic study of this lymph node showed greatly enlarged lymph follicles with some confluence. The individual cells making up these follicles possessed a curious pleomorphism. They invaded the capsule and the surrounding fat. The diagnosis was "malignant lymphoblastoma." A preauricular lymph node which had become enlarged in 1943 disappeared completely following high voltage roentgen therapy. Late in February, 1945, a growth on the hard palate was resected. Sections showed fibrous tissue moderately infiltrated with lymphocytes, a few plasma cells, eosinophiles, and occasional polynuclear neutrophils. There were no Reed-Sternberg cells. The interpretation was chronic granulomatous inflammation. On April 3, 1945, a tumor was removed from the region of the right scalenus muscle, sections as studied by Dr. S. B. Pessin showed a disordered lymph gland with the normal architecture completely destroyed. The predominant cells were large and medium-sized lymphocytes, containing a vesicular nucleus with one or two distinct nucleoli. There was a small amount of fibrosis in some areas and considerable delicate reticulum. Occasional mitotic figures were seen. The diagnosis was reticulum cell lymphosarcoma.

Early in November, 1945, the patient noted a lump in the right groin which brought her to the State of Wisconsin General Hospital on March 6, 1946. Eight days later, the node from the right groin was removed and at the same time a 3 cm node from the thyroid was excised. General examination at this time showed no other lymph node enlargement nor enlargement of the spleen and liver. There was no anemia, the total leukocyte count was 4,300 with a normal differential count. The section from the thyroid showed closely spaced giant follicles, but some fusion of follicles had taken place so that the picture resembled a fully developed reticulum cell lymphosarcoma. The diagnosis of Dr. Walter Jaeschke was follicular lymphoblastoma.

The patient has remained well to date and her case has been followed in the outpatient department.

*Comment* This is a rather amazing case with the evidence, quite reasonably substantiated, of multiple malignancies. The reports of the several lymph-node studies might well leave one confused, and perhaps the pathological findings were

not the same in the original studies as in the last.\* This emphasizes, it seems, the close relationship between follicular lymphoblastoma and other malignant disease (lymphosarcoma). The last biopsy was sufficiently characteristic to justify the inclusion of the case in this series.

*Case 5 J G* This patient, a white male of 82, was admitted to the State of Wisconsin General Hospital on April 24, 1945. He had an enlarged right tonsil, which had first been noticeable four months before and had been progressively growing. There was no pain nor bleeding, and although the patient was conscious of the mass, it caused no real difficulty in eating.

The patient was very deaf and almost blind. The teeth were very carious. The enlarged right tonsil protruded well into the midline and filled half the throat. The mass was irregular, hard, and nontender. There was no enlargement of the lymph nodes, nor of the spleen and liver.

The blood count showed a hemoglobin of 11.6 Gm (70 per cent), erythrocytes 3,600,000, the leucocyte count was normal. A roentgenogram of the chest showed no enlargement of mediastinal nodes.

The tonsils were removed surgically. The left was fibrous. The right tonsil was 4 x 2.5 x 2 cm. There was loss of usual architecture, although a number of large giant follicles could faintly be made out. Throughout the sections there were occasional endothelial cells, numerous small round cells closely resembling lymphocytes, and occasional mitotic figures. The interpretation of Dr. W. D. Stovall was follicular lymphoblastoma. The patient died of bronchopneumonia six days after the operation. Post-mortem examination was not permitted.

*Comment* The disease in this instance involved, so far as could be determined, only the tonsil. Baehr, Klemperer, and Rosenthal<sup>11</sup> observed that in their cases the tonsils and lymphatic apparatus of the gastrointestinal tract had not been involved. Baggenstoss and Heck<sup>10</sup> report 2 cases with tonsillar involvement and two other reports describe nasopharyngeal tumors with the histologic picture of follicular lymphoblastoma in which neither the lymph nodes nor spleen<sup>17, 20</sup> were grossly involved. Tonsillar involvement with other types of lymphosarcoma is by no means rare.

*Case 6 S W*, a male Chippewa Indian 60 years old, was admitted to the Wisconsin General Hospital on April 29, 1943, complaining chiefly of weakness. He had been in good health until six weeks previously, when he began to suffer from weakness which became progressive. Four weeks before admission, he had gone to his local doctor, and a week later he noted swelling of the penis and scrotum which progressed. Three days before admission he developed swelling of the left leg. In response to questioning he revealed that for two years he had noted enlarged nodes in the left inguinal region and similar nodes on the right for four or five months. During the past six weeks, nodes in the axillary and cervical region and an abdominal mass became apparent. There had been a nonproductive cough, and for seven weeks intermittent tarry stools. The patient had had pleurisy at the age of 21.

Physical examination showed a moderately well-nourished man. Scattered in the cervical, supraclavicular, and axillary regions were moderately firm, discrete nodes 1 to 2 cm in size, and there was a tiny left epitrochlear node. There were similar nodes, 2 to 3 cm in diameter, in the inguinal regions, and a large firm mass filled most of the left side of the abdomen. Expansion of the left lung was limited, and there was dullness at the left base. The liver and spleen were not palpable. The penis, scrotum, left lower extremity to the mid-thigh, and the right foot were moderately edematous.

Laboratory studies showed a hemoglobin of 13.3 Gm (80 per cent), erythrocytes 4,250,000, leukocytes 6,700 with neutrophils 64 per cent, lymphocytes 29 per cent, monocytes 2 per cent, and eosinophils 5 per cent. Blood Wassermann was negative, Hanger's cephalin-cholesterol flocculation test was negative. Roentgenogram of the chest showed atelectasis at the left apex with displacement of the trachea to the left. There was extensive fibrosis and some calcification of the pleura with a small left pleural effusion.

\* The biopsy section of April 3, 1945, has been reviewed and the diagnosis of reticulum cell lymphosarcoma confirmed.



The mediastinal shadow was widened, and radiating areas of infiltration extended into the field of the lower left lung. X-ray of the colon following barium enema showed marked narrowing of the sigmoid apparently due to an extrinsic mass. Gastrointestinal roentgenograms showed that the esophagus deviated sharply to the left in its upper portion and that the trachea was displaced in consequence of the fibrotic and calcified pleura of the left apex. The stomach was displaced upward because of abdominal masses and fluid. The duodenal loop was large and rounded, probably as a result of a mass of nodes about the head of the pancreas. Biopsies were done of an inguinal node, of an epitrochlear node, and later of an axillary node. Large hyperplastic follicles were noted, which almost crowded out the pulp lymphocytes. Dr. W. D. Stovall interpreted these nodes as malignant giant folliculoma (follicular lymphoblastoma).

The patient, after a period of twenty essentially afebrile days in the hospital during which time he received eight treatments of 200-r each of roentgen therapy ( $HVL_{Cu} = 2.4 \text{ mm}$ , distance 50 cm) to the anterior and posterior mid-abdomen, was discharged on May 19. He had been seen in the hospital and in the outpatient department at intervals of about three months to December 11, 1946, his hospital admissions totaling ten. He had continued to have quite general enlargement of the lymph nodes, and on his second admission the spleen and liver were enlarged. At times superficial nodes were as great as 5 to 6 cm in diameter. Roentgen therapy to various sites had been given with each admission. On several occasions, abdominal paracentesis, resulting in the removal of as much as 3,000 cc of creamy, foul smelling fluid, was done. Temporary improvement followed each course of therapy. In August, 1945, when he was admitted for the seventh time, he complained of having suffered shooting pains in the legs for ten days, making it impossible to walk. For four days previously, he had walked dragging his feet. He could move his toes slightly. Examination at this time showed scattered superficial nodes 1.5 to 3 cm in diameter, persistence of pulmonary changes, and marked weakness of the legs, though he could move them a little in bed. The knee jerks were present, but Achilles reflexes and abdominal reflexes were absent. Babinski and confirmatory signs were present bilaterally. There was tenderness over the spine at the level of the sixth and seventh dorsal vertebrae. Spinal tap was done and there was evidence of block.

Laboratory studies showed a hemoglobin of 10.7 Gm (69 per cent), erythrocytes 3,030,000, lymphocytes 4,240 with a relative lymphocytosis. Spinal fluid showed a negative serology, gold sol 001233 2100, no cells, sugar 63 mg, protein 415 mg per 100 cc of blood. X-ray of the spine showed no alteration in the vertebra, but roentgenogram of the chest showed distinct increase in the mediastinal mass with metastatic nodules in the right pleura. The patient received four roentgenographic treatments of 200-r each to the anterior and posterior mediastinum and was discharged as improved. He gradually regained some strength in his legs but in October, 1945, was still unable to walk. On this admission the hemoglobin was 8.2 Gm (50 per cent), erythrocytes 2,350,000. The chest x-ray showed a marked decrease in the width of the mediastinal mass but an increase in the size and number of the metastatic nodules on the field of the right lung. Further roentgenotherapy was administered to the mediastinum.

The patient was next admitted on August 1, 1946, complaining of pain and weakness about the right knee and in the right leg which was relieved by rest. He was thin and pallid. There were scattered enlarged lymph nodes 2 to 4 cm in diameter. There was a 2 x 2 cm tender nodule on the medial side of the right femur just about the knee. Roentgenogram of the right femur at the junction of the middle and lower thirds showed an osteolytic lesion involving the shaft of the femur for a distance of 7 to 10 cm. A large central rarefaction extended almost through the cortex laterally, and there were a number of smaller rarefactions which were intercommunicating. Pathologic fracture was thought to be imminent. The patient was placed in a hip spica cast and seven treatments of 150-r each in air ( $HVL_{Cu} = 1.05 \text{ mm}$  to 2.40 mm, distance 50 cm) were administered to the femur through a window in the cast. As the right testis was enlarged, irregular, and hard, and was believed to be involved by tumor, a small amount of therapy was directed to it, also.

The patient next entered the hospital on November 26, 1947. The pain in the right lower extremity persisted but was less severe and less constant. The patient was thin and pallid. A few superficial nodes were palpable. There was tenderness of the left fifth rib. The cast was still in place on the right lower extremity. Hemoglobin at this time was 11.3 Gm (70 per cent), erythrocytes 4,320,000, leukocyte count 2,650 with 56 per cent neutrophils and 8 per cent eosinophils. The basal metabolic rate was plus 13 and plus 15. Serum proteins were albumin 4.9 Gm and globulin 2.1 Gm per 100 cc. Roentgenogram of the chest showed no evidence of recurrence of the mediastinal mass. X-ray of the right femur showed increased destruction of the cortex in the distal portion of the femur. Biopsy of a right epitrochlear node

which was made on December 3 showed the outlines of giant follicles (three and a half years after the inguinal biopsy) which were interpreted by Dr Walter Jaeschke as follicular lymphoblastoma. After five treatments of 200-r each in air (H V L  $\text{cu} = 2.4 \text{ mm}$ , distance 50 cm) to the femur through a window in the cast, the patient was discharged. The last admission was April 17, 1947. The patient at this time was emaciated. Biopsy of the bone lesion showed several small cellular foci composed of closely packed



FIG. 3. LATERAL AND A. P. VIEWS OF THE FEMUR, CASE NO. 6

small cells, apparently lymphocytes. There were no follicles. The interpretation of Dr. Walter Jaeschke was necrotic lymphoid tissue.

The patient died April 25, 1947. The autopsy was done by Dr. John W. Harman of the Department of Pathology, and he reported that the autopsy demonstrated a large, firm, diffuse, greyish-white retroperitoneal mass which included both adrenal glands, infiltrated the pancreas, was attached to the undersurface of the liver and surrounded and constricted the pelvi-ureteric junction of the left kidney. There were several discrete nodules of similar tissue in the liver (see fig. 4). The only enlarged lymph nodes seen were the left epitrochlear and right external iliac. The spleen weighed 250 Gm. Microscopically the

retroperitoneal mass, liver nodules, and enlarged lymph nodes had a predominantly follicular structure. The large follicles were widely separated by diffuse areas of small lymphocytes and were composed of similar cells themselves. By reticular stain the follicular structure was accentuated, each follicle was surrounded by a zone of compressed reticulin fibers. In all sections the cell type was almost exclusively



FIG. 4. SECTION OF THE LIVER SHOWING NODULES FROM THE AUTOPSY, CASE No. 6

small lymphocytic, only rare clasmotocytes were seen. The splenic structure was normal, the follicles were few, small, and widely separated by the pulp. Diagnosis: malignant follicular lymphoma.

*Comment.* A male Indian with follicular lymphoblastoma which may have begun in 1941 and was diagnosed in 1943, evidencing widespread disease with probable involvement of the spinal cord and definite involvement of bone with resultant destruction. The architecture of the lymph nodes had changed, but some char-

acteristics of follicular lymphoblastoma persisted in the biopsy done in December, 1946, although the patient's condition was poor and the disease far advanced. The development of a bone disease such as this strongly suggests that we are dealing with a malignant tumor such as a lymphosarcoma. Finally, at autopsy, four years after onset of the illness, there was still the characteristic pathologic change of the follicular lymphoblastoma.

### DISCUSSION

The six cases herein described represent varying symptomatic states dependent upon varying sites of the lesions and emphasize the protean manifestation of follicular lymphoblastoma. Two cases, No. 5 and No. 6, present previously rarely reported or unrecorded lesions.

Analysis of these cases tends to confirm observations made by Baehr, Klemperer, and Rosenthal<sup>11</sup> and by Baggenstoss and Heck.<sup>10</sup> The disease tends to, but does not invariably, pursue a relatively slow course. The onset is ordinarily insidious. Pleural effusion was prominent, occurring in 3 of the 6 cases. Ascites was demonstrated in case No. 6. Hypochromic anemia was almost invariably present, when the patient was seen late, rarely so if seen early. The leukocyte counts showed nothing characteristic early or late. Cachexia was unusual until late in the disease. This is quite characteristic of other types of lymphosarcoma, however, whereas anemia appears relatively early in Hodgkin's disease. The course in those of our cases followed for long periods certainly suggests that one is dealing with a neoplastic process, malignant in character. Bone destruction, prominent in one case, No. 6, and involvement present in another, No. 2, further suggests the malignant character of follicular lymphoblastoma. In a word, our observations, although not extensive, tend to confirm the conclusion of Baehr, Klemperer, and Rosenthal<sup>11</sup> and Baggenstoss and Heck<sup>10</sup> that this is a form of lymphosarcoma, and that, although readily amenable to roentgen therapy it is not ordinarily curable by this type of therapy, as has been suggested.<sup>31</sup>

This series does not enable one to form an opinion as to the duration of the disease, but general opinion holds that the course is slower than with the other forms of lymphosarcoma. Of our cases, No. 6 has been ill for four years and may have had lymph node involvement for six years. Case No. 1, whom we observed from early in her course to almost the end, survived about eighteen months. Baggenstoss and Heck<sup>10</sup> have discussed well the pathologic differentiation of follicular lymphoblastoma and lymph node hyperplasia of inflammation and our observations add nothing in this regard. Evans<sup>32</sup> has listed the pathologic differences of lymphadenitis of secondary syphilis and follicular lymphoblastoma.

### CONCLUSIONS

Six cases of follicular lymphoblastoma are here reported. They demonstrate a variety of pathologic lesions with a characteristic histologic picture with variable symptoms and signs. Tonsillar involvement was unique as the sole demonstrable lesion in one. Bone involvement occurred in two.

Study of these 6 cases leads us to believe that we are dealing with a clinical and pathologic entity and a malignant tumor, one which is usually highly sensitive to roentgen therapy, but ordinarily recurrent and progressive

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## THE SIGNIFICANCE OF MEGAKARYOCYTES IN THE PERIPHERAL CIRCULATION

By SIR LIONEL WHITBY

SOME of George Minot's earliest publications were concerned with platelets and megakaryocytes and the significance which should be attached to an increase of the former or the presence of the latter in the circulation. In a paper entitled "Megakaryocytes in the peripheral circulation," Minot (1922) pointed out that an increase in circulating platelets was usual whenever megakaryocytes were present in the peripheral blood, with the exception of myelogenous leukemia in which, despite megakaryocytes, the platelets might still be normal and even decreased. Minot's general observation was that whenever megakaryocytes or fragments were found there was usually other evidence of a grave disturbance of marrow function, as suggested by the simultaneous presence of immature cells of either the white or red cell series, or both. This was an early conception of the phenomenon nowadays known sometimes as leuko-erythroblastic anemia or, sometimes as a leukemoid blood picture caused by a nonleukemic disease.

As to myelogenous leukemia, in which primitive leukocytes (and usually red cells) were already present, Minot suggested that the appearance of megakaryocytes might be a sign of an acute exacerbation of the disease, he observed that when megakaryocytes appeared, the blood picture often changed from myelocytic to the terminal myeloblastic predominance.

Minot's general deductions were that since neither myeloblasts, nor nucleated red cells, nor megakaryocytes are found normally in the blood stream, then the finding of any or all of these cells pointed to a serious alteration in the mechanism regulating the emergence of cells from the bone-marrow into the circulating blood, in such cases, the marrow was subject to great strain, the pathologic process underlying the regulating mechanism was varied, since the immature cells occurred in the blood not only when there were 'structural changes in the marrow,' as in myelogenous leukemia, but also with other changes, 'probably functional, as in pneumonia and sepsis.'

Nowadays, one could add a number of other diseases which bring about 'structural changes in the marrow,' to Minot's example of myelogenous leukemia. An ability to hint at a correct diagnosis in these other diseases, which include carcinomatosis and Hodgkin's disease of bone, myelomatosis, osteosclerosis and myelosclerosis, Cooley's anemia and lipomatosis of the bone marrow (Rosenthal and Erf, 1943) can make or mar a hematologist, who must always be on the alert when he is confronted with a leukemoid blood picture.

Minot's suggestion of the significance of megakaryocytes was, therefore, an observation of fundamental practical value.

In a later publication, Minot and Buckman (1923) drew attention to the fact that megakaryocytes may share in the hyperplastic process of both leukemia and erythremia (polycythemia vera) and that in the former condition, the megakaryo-

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cytes may sometimes appear to be more involved than the leukocytes, so that the blood might indeed be flooded with megakaryocytes and their derivatives, the platelets. For a time, the leukemic process might seem to be almost restricted to the megakaryocytes, even as frank erythremia or leukemia cause varying degrees of pathologic activity of myeloid or erythroid tissue in conformity with a definite type. This paper has sometimes been quoted as suggesting a megakaryocytic type of leukemia, but such is a misrepresentation of Minot and Buckman's views, which go no further than to say that the disease process "appears to be" confined to the megakaryocytes. When taken in conjunction with Minot's (1922) earlier paper the significance of megakaryocytes in erythremia would seem to be a hint that the disease was in process of transition to some complication, possibly to the malignant erythro-leukemic form or to the terminal stage of a "spent" hyperplastic marrow which is becoming sclerosed. This last, as I shall presently show, is the more probable explanation, in view of the frequency with which megakaryocytes and abnormal platelets are found in the blood in myelosclerotic conditions.

Minot and Buckman (1925) followed up their first paper by another on "The Blood-platelets in the Leukemias," in which they concluded that the platelet count yielded useful knowledge for guiding treatment and for appraising the state of the leukemic patient. They observed that the platelets, especially in myelogenous leukemia, might be greatly increased or much reduced, whereas in the acute leukemias and the chronic lymphatic type it was usual to find the platelet count below normal. They noted that petechiae and hemorrhages were often associated with platelet decrease, and that hemorrhages, though not petechiae, might be found in chronic myelogenous leukemia, even when the platelets were greatly increased.

These three of Minot's early papers emphasize four important points, of which some are nowadays accepted as commonplace, though others are not widely known.

- 1 That megakaryocytes in the circulation are an indication of a serious disturbance of the bone-marrow. The fact must be taken into account when framing a prognosis.

- 2 That the bone-marrow disturbance is commonly leukemic in origin, but not always so. In the latter case, there is frequently a leuko-erythroblastic anemia, and the causes of such must be considered.

- 3 That when megakaryocytes appear in the circulation in leukemia or erythremia, they indicate of an impending change in the character of the disease.

- 4 That the hemorrhagic manifestations of leukemia are not due entirely to reduction in platelets.

#### MEGAKARYOCYTIC LEUKEMIA

Boros and Korényi (1931) described a case which they designated as megakaryoblastic leukemia. The case was severely anemic and had a leukocyte count of the order of 200,000 cells per cu. mm., among which the most primitive cells were described as large mononuclear leukocytes 10-25  $\mu$  in size, megakaryocytes, typical and atypical, complete and fragmented, were numerous in the blood. The clinical course of the disease as well as the postmortem description, suggest a diagnosis of



an acute termination of myeloid leukemia. Indeed, there can be little doubt that Boros and Korényi were observing no more than what Minot had described years before—the appearance of megakaryocytes in the circulation in myelogenous leukemia.

The literature also contains a number of reports under such descriptive names as “chronic nonleukemic myelosis” (Hickling, 1937, Carpenter and Flory, 1941), “aleukemic megakaryocytic myelosis” (Favre et al., 1934) and “myeloid megakaryocytic hepato-splenomegaly” (Downey and Nordland, 1939). Most of the cases described under these various titles have exhibited a leuko-erythroblastic anemia, with megakaryocytes and their fragments in the peripheral blood (as much as 26 per cent of all nucleated cells in Carpenter and Flory’s case). The spleen and liver have been enlarged, but seldom the lymph glands. Sections of the spleen, the bone marrow and even the liver, as well as sites of extramedullary hemopoiesis have shown numerous megakaryocytes. Nearly all such reports concern cases of myelofibrosis, and the frequency with which the megakaryocytic phenomenon is prominent in this condition has been well presented and illustrated in the account given by Rosenthal and Erf (1943) of 17 cases of myelofibrosis and one of osteopetrosis (Albers-Shonberg disease). The megakaryocytic tissue in the spleen and other organs arises from myeloid metaplasia, sometimes the process has been so prominent that authors have put forward the idea of a megakaryocytic leukemia. This cannot be accepted on the evidence presented. It would seem that myelofibrosis is the fundamental factor in producing this megakaryocytic type of metaplasia, whether the underlying cause of the fibrosis be idiopathic, or the “spent” stage of polycythemia, or even myeloid leukemia and other invasive conditions (carcinomatosis, etc.) of the marrow. In practice, whenever megakaryocytes, fragments, giant or bizarre forms of platelets, or even gross platelet increase, are found in the circulation, the question of myelofibrosis should be considered. Other small practical points can each or severally support the diagnosis. These include the evidence afforded by the other features of the blood count, by sternal puncture, and more especially by sternal biopsy, by radiologic examination of the bones with suitable controls of the same age group and, if thought necessary, by splenic puncture.

The result of sternal puncture is usually disappointing on the positive side. This in itself should raise suspicion when the accomplished operator is unable to obtain a satisfactory marrow sample by puncture, and especially if the bone feels gritty on puncture. The sample usually contains few cellular elements derived from the marrow, but either giant platelets or megakaryocytes are highly suggestive. In such cases, sternal biopsy should be performed. With a section, the fibrous nature of the marrow is revealed, and the lack of cellularity often associated with numerous megakaryocytes provides a diagnostic picture. Radiologic examination of the bones occasionally shows mottled rarefactions or irregular condensations in the cortices, which need to be compared with appropriate controls taken at the same time and with the same exposure (Hynes, 1940). Splenic puncture sometimes reveals the characteristic myeloid metaplasia with many megakaryocytes.

The following is a brief summary of a case recently referred to me for adjudication

by The Ministry of Pensions, it illustrates the confusion which may arise in patients who exhibit splenomegaly with leuko-erythroblastic anemia—a confusion which becomes more confounded under service conditions, when the patient moves from one hospital to another

### REPORT OF CASE

Sergeant B E P was examined on re-enlistment in 1940, at the age of 38, and placed in Category A1. He had an uneventful service until, during the campaign in N W Europe in 1945, he reported sick with vague pain in the left upper abdomen. He was found to have a spleen enlarged to  $2\frac{1}{2}$  fingers breadth below the costal margin, the liver was not enlarged. The blood count, performed under field conditions, was reported as Hb 13 Gm per cent, red cells 4.5 million per cu mm, leukocytes 6.5 thousand per cu mm, with no exact differential, though the report stated that 250 cells were abnormal, including myeloblasts, myelocytes, metamyelocytes and some cells of 'unknown origin', there were also a few early and intermediate normoblasts.

The man was evacuated from Europe and reinvestigated in England. The record of the blood count was Hb 13.8 Gm per cent, red cells 4.4 million per cu mm, leukocytes 6.2 thousand per cu mm, with 3.6 per cent myelocytes, 0.4 per cent myeloblasts, 0.8 per cent metamyelocytes, 2 megakaryocytes and 0.8 normoblasts per 100 leukocytes. Investigations included the exclusion of syphilis and glandular fever and a diagnosis of aleukemic leukemia was made. A blood count a month later was approximately the same, save that many giant platelets were observed. At the same time, a sternal puncture was performed, which showed essentially the same cell content as the blood, except for a slightly higher proportion of myelocytes and larger numbers of giant platelets and 2 megakaryocytes per 100 nucleated cells.

The patient then had a number of medical boards, where he was labelled ? leukemia, ? Hodgkin's disease, ? Banti's syndrome. At one of the boards, it was noted (without comment) that the patient was high colored and had a blood pressure of 160/90. He was discharged from the Army shortly afterwards. He worked as male nurse for two years, and was then re-examined on account of his pensions appeal. He stated that he had gone down hill a little, but was reasonably well. His blood pressure was 180/110, and there was some left ventricular hypertrophy, the spleen was enlarged to three fingers breadth below the costal margin, and the liver was easily palpable. The blood showed no increase in the anemia, the leukocyte count was 6.3 thousand per cu mm, with 8.5 per cent of abnormal cells, of which 0.5 per cent were myeloblasts, 2.5 per cent unidentified cells, and the remainder either myelocytes or metamyelocytes, there were 3 megakaryocytes and 4 normoblasts per 100 leucocytes.

Thus, during the intervening two years, the character of the leuko-erythroblastic anemia had not altered significantly, nor, indeed, had the clinical state greatly deteriorated. The spleen had become more enlarged, the liver had become palpable and the blood pressure had risen. The true nature of this man's disease, which might have been suspected from the outset by reason of the hematologic findings, is amply confirmed by the subsequent history and later records.

Most of the points relative to the title of this article, which is virtually the title of Minot's original (1922) paper, can be extracted from the above case record, and expressed as a

### SUMMARY

1 Leuko-erythroblastic anemia, with leuko-erythroblastosis, rather than anemia, when associated either with the presence of megakaryocytes or giant platelets in the circulation, is very suggestive of the myeloid metaplasia so commonly found with myelofibrosis.

2 Associated splenomegaly with subsequent slow progress to hepatomegaly and a tendency to hypertension are confirmatory clinical features

3 Sternal puncture may or may not confirm the diagnosis, but if the specimen contains megakaryocytes, the fact is highly significant. Difficulty in piercing the bone or in obtaining a satisfactory marrow sample are points in favor of a myelofibrosis, which should be confirmed by the histologic examination of a trephined specimen

4 Controlled radiologic examination of the bones is sometimes of value in establishing a diagnosis in the idiopathic disease

5 A similar blood picture may occur with polycythemia vera, with myeloid leukemia and, occasionally, with other conditions in which invasion of the bone-marrow occurs. In polycythemia vera the finding suggests a terminal phase of exhaustion, in myeloid leukemia, likewise, the megakaryocytic phenomenon is usually an ominous sign of the terminal phase

6 When megakaryocytes are found in the circulation a diagnosis of myelofibrosis should always be considered

This short and simple article, which draws attention to some of George Minot's early work, brings with it the greetings of the entire staff of The Cambridge University Medical School to a great physician

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# THE ACTIVE PRINCIPLE IN THE LEUKOCYTOSIS-PROMOTING FACTOR OF EXUDATES

By VALY MENKIN, M D

THE earlier studies of the writer have demonstrated that the leukocytosis accompanying many inflammatory processes is referable to the liberation at the site of inflammation of a factor closely associated with the pseudoglobulin fraction of exudates <sup>1, 2, 3</sup> The factor per se offers a reasonable explanation for the mechanism of leukocytosis with inflammation This factor not only induces a discharge of immature leukocytes into the circulating blood,<sup>1</sup> but it also is capable of producing a hyperplasia of granulocytes and of megakaryocytes in the bone marrow <sup>4</sup> The factor (abbreviated as the LPF) is active on human beings, thus suggesting possible clinical application <sup>5</sup> It has been found to be active on guinea pigs This animal may well serve as a convenient assay animal <sup>6</sup> In as yet unpublished studies, it has been found that the LPF reinforces the leukocytosis caused by an already existing inflammation This may be significant in the usage of the material in clinical cases with inflammatory processes It is quite conceivable that the factor may be utilized as an adjunct to the antibiotics

The leukocytosis-promoting factor has always been recovered in the form of a pseudoglobulin Recent studies in association with Dr G Cooper and Mr M L Dillon at Duke University suggest that the LPF seems to be distributed primarily between the  $\alpha_1$  and  $\alpha_2$  globulins of exudates In the present communication evidence is furnished which suggests that the active group in the pseudoglobulin molecule of exudates is a relative simple polypeptide

## EXPERIMENTAL

The leukocytosis-promoting factor (LPF) utilized in the following observations has been obtained from pleural exudates in dogs previously injected with 1.5 cc of turpentine as described in the past <sup>7</sup> The scheme of extraction can be briefly restated by referring to the diagram on p 940

The material when freshly obtained appears as a fluffy, white powder which is extremely soluble in an aqueous medium It is active in dogs, inducing a discharge of immature white cells into the circulation

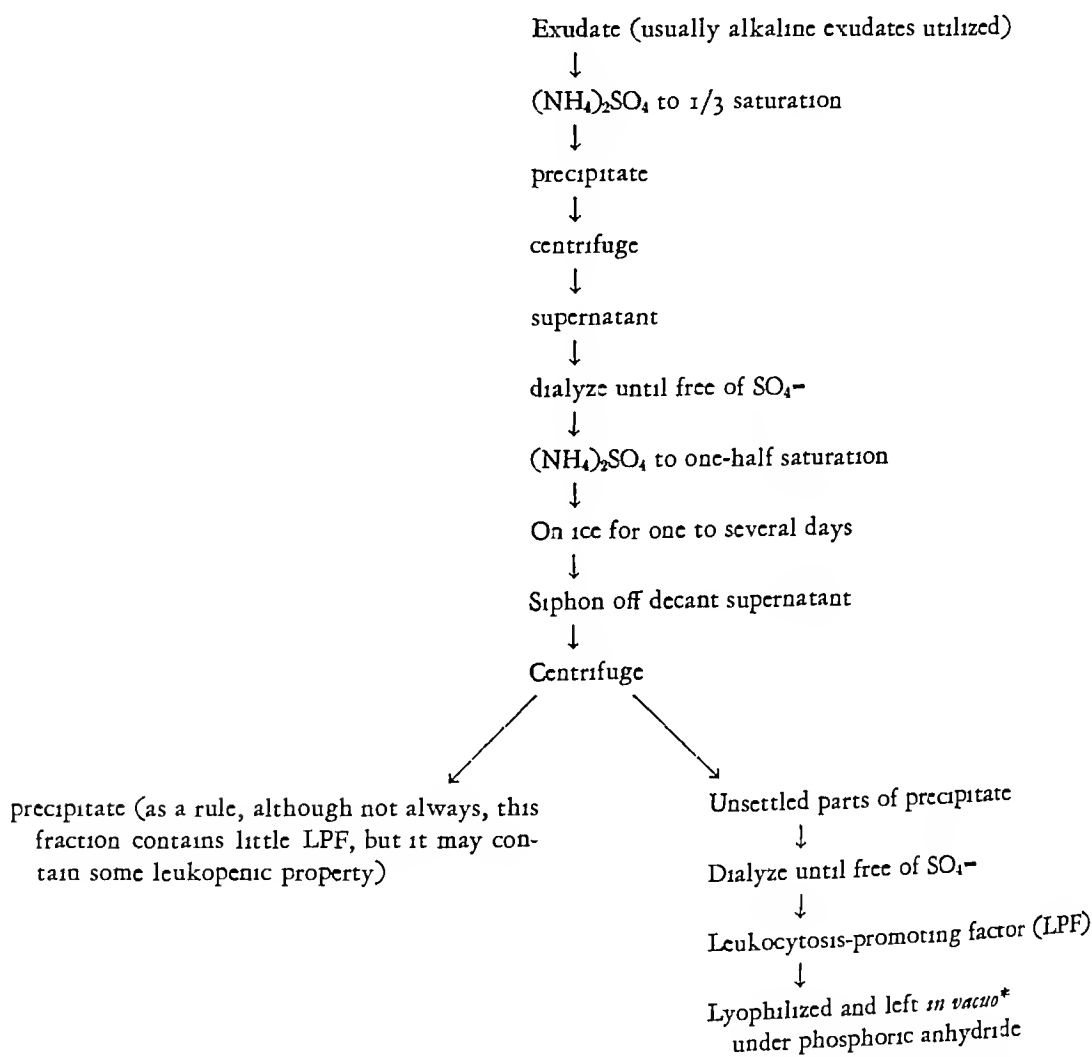
After several months, a curious change occurs in the material It seems to lose its solubility, becoming quite insoluble in physiologic saline, and at the same time the material loses its biologic activity It now has either no or little activity in causing a rise in the number of circulating leukocytes It seems as if a spontaneous

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The aged samples of leukocytosis-promoting factor used in this study were for the most part prepared at Duke University School of Medicine Aided also in part by a grant from the Bristol-Myers Company of New York

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denaturation has occurred. If the material is then centrifuged, the now insoluble part of the precipitate may even induce a leukopenic effect in contrast to the original leukocytosis-promoting property which it possessed. This, however, is not consistently true. At times it has either no activity, or at most a weak activity. On the other hand, if the supernatant phase of the insoluble aged LPF is injected



into the blood stream of a normal dog, considerable activity is obtained. The supernatant fraction induces a rise in the number of circulating leukocytes. It appears as if aging of the LPF causes a spontaneous denaturation into a relatively inactive and insoluble part. It splits off the active principle in the form of soluble component. The data of several such experiments appear in table I. It is clear that when 10 to 20 milligrams of aged LPF (3-6 months old) is treated with about 10 cc of saline, stirred, and centrifuged, the supernatant part yields considerable activity when injected into dogs. There is an increase of about 64 per cent in the number of circulating leukocytes (table I). The evidence indicates that the active

\* Determination on three samples of LPF have yielded a recovery on the average of 12.8 milligrams of LPF per cc of exudate.

principle is liberated *in toto* as a soluble component from the now insoluble and aged sample of leukocytosis-promoting factor. The course of one such experiment is shown in figure 1.

TABLE 1—Effect of a Soluble Fraction Derived from Aged LPF (3–6 months old) on the Leukocyte Level

Dog No	Amount of original LPF from which soluble fraction derived	Basal no. of white blood cells	Maximum no. of white blood cells within 3–6 hours following administration of material
	mg	cu mm	cu mm
3-T	10	18,850	30,250
3-T	14.5	19,000	46,125
8-D	14	11,000	27,175
5-T	13.5	16,975	24,050
6-T	14	19,000	22,250
8-T	11.5	16,950	27,300
8-T	17	18,700	20,100
9-T	10	16,000	25,700
10-T	20	9,800	16,850
11-T	20	14,700	24,450
Average		16,097	26,425*

\* Percentage increase in leukocyte level = 64.2%

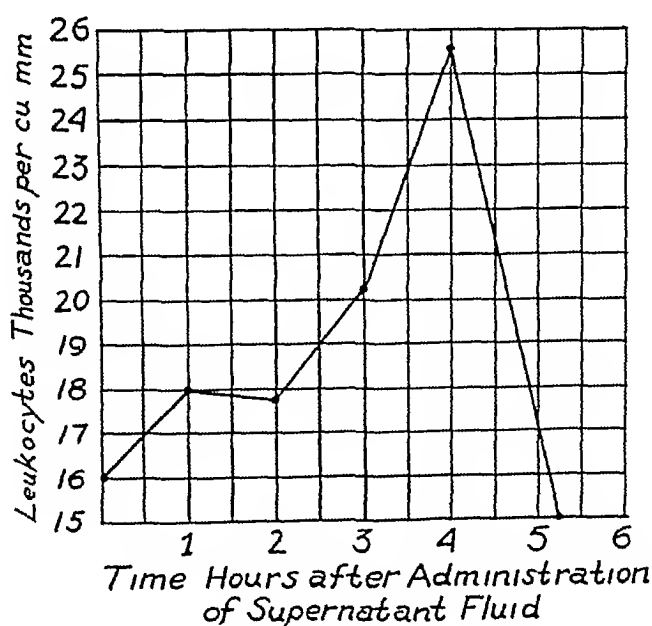


FIG. 1. EFFECT OF SUPERNATANT FRACTION FROM AN OLD SAMPLE OF LPF ON THE LEUKOCYTE LEVEL  
Dog 9-T. Supernatant from 10 milligrams of 5 months old LPF employed.

In view of this observation, it became of interest to determine whether a proteolytic enzyme would inactivate the leukocytosis-promoting factor when freshly recovered as a soluble pseudoglobulin from exudates. The LPF was extracted from exudates of dogs, as described above. Various quantities of the factor in the fluid

state were treated with crystalline trypsin in amounts varying from a mere pinch of the enzyme to 2 milligrams. The length of incubation with the LPF was also variable, lasting from about one hour to over twelve hours. The treated LPF failed to be inactivated by tryptic digestion. The observations are assembled in table 2. It is quite clear that the addition of trypsin has failed to inactivate the factor. Following such digestion the injection of the treated material still caused a rise of 111.5 per cent in the number of circulating leukocytes (table 2). The course of an experiment is graphically shown in figure 2. Trypsin is known to hydrolyze complex proteins, and also the products of peptic digestion to the peptide stage.<sup>8</sup>

TABLE 2 — *Effect of Tryptic Digestion on the Activity of the Leukocytosis-Promoting Factor*

Dog No	Material injected	Basal no of white blood cells	Maximum no of white blood cells within 2-6 hours following injection of treated LPF with enzyme
		<i>cu mm</i>	<i>cu mm</i>
11-T	13 cc LPF + pinch crystalline trypsin	12,100	24,600
12-T	18 cc LPF incubated overnight with trypsin	7,225	11,300
16-T	20 cc LPF incubated with trypsin 2 hours	12,275	25,900
9-T	18 cc LPF incubated with trypsin 2 hours	10,200	30,900
17-T	17 cc LPF incubated 2 hours with 2 mgm trypsin	6,300	8,950
20-T	10 cc LPF incubated 1 hour and 5 minutes with 1 mgm crystalline trypsin	10,900	24,100
9-T	10 cc LPF incubated 1 hour and 25 minutes with approximately 1 mgm crystalline trypsin	10,750	21,750
Average		9,964	21,071*

\* Percentage increase in leukocyte level = 111.5%

On the basis of the foregoing evidence, it is conceivable that the leukocytosis-promoting factor is not a protein at all, and therefore it remains intact when subjected to the influence of a proteolytic enzyme.

Yet when the newly obtained and active leukocytosis-promoting factor is heated for 30-35 minutes at 100 C, the whole molecule appears to be denatured and the LPF is likewise inactivated. The results of these experiments are summarized in table 3. When the LPF is exposed to such temperature, the material is completely inactivated. The LPF now yields a rise of 6 per cent in the number of circulating leukocytes in contrast to its original activity of 104 per cent (table 3). The course of such an experiment is illustrated in figure 3.

These observations simply indicate that the LPF is thermolabile, but neverthe-

less that it may be merely associated with the pseudoglobulin molecule without being an actual part of it

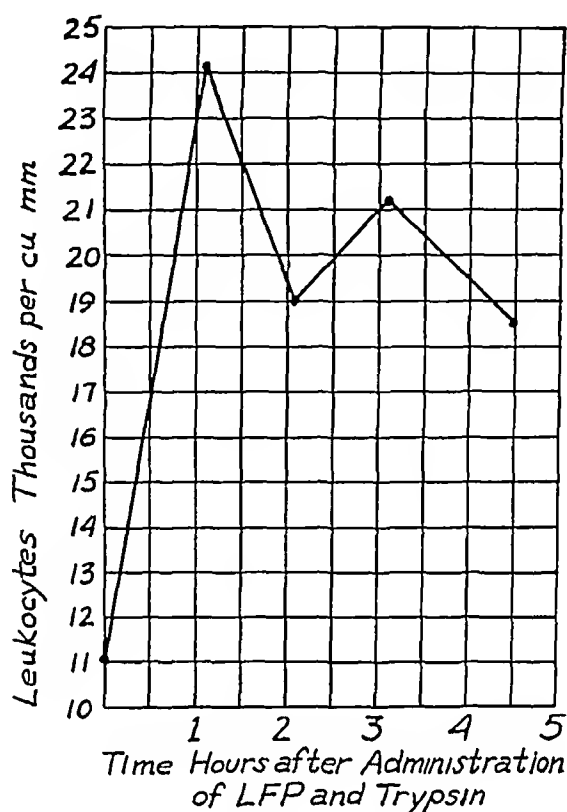


FIG 2 THE FAILURE OF TRYPSIN TO INACTIVATE THE LPF

Dog 20-T 10 cc of LPF + 1 milligram of trypsin incubated for 1 hour and 5 minutes before administering to the animal

TABLE 3 —Effect of Heat (at 100 C for 30-35 minutes) on the Activity of the Leukocytosis-Promoting Factor

Experiment No	Amount of LPF used	Basal white cell count in experiment with unheated LPF	Maximum white cell count 3-6 hours following administration of unheated LPF	Basal white count in experiment with heated LPF	Maximum white cell count 4-6 hours following administration of heated LPF
	mg	cu mm	cu mm	cu mm	cu mm
1	20	8,500	17,400	9,375	9,550
2	19	9,425	19,500	7,350	8,350
3	17	10,575	16,550	8,825	8,400
4	25	11,800	27,950	9,425	10,600
5	23	11,000	23,250	13,275	14,150
Average		10,260	20,930	9,650	10,210

Percentage increase in leukocyte level with unheated LPF = 104%

Percentage increase in leukocyte level with heated LPF = 6%

Such an interpretation is, however, not consistent with subsequent facts. When now the active supernatant or soluble fraction obtained from aged LPF is evaporated to dryness on a steam bath, its activity remains essentially intact. Evapo-



ration of the active supernatant material even over a steam bath fails to inactivate the principle. These experiments are collected together in table 4. When such brittle, dried material obtained by evaporation over a steam bath is heated for 30-40 minutes at 100 C, the active principle fails to be inactivated (table 4). The active supernatant material when evaporated to dryness forms brittle flakes which

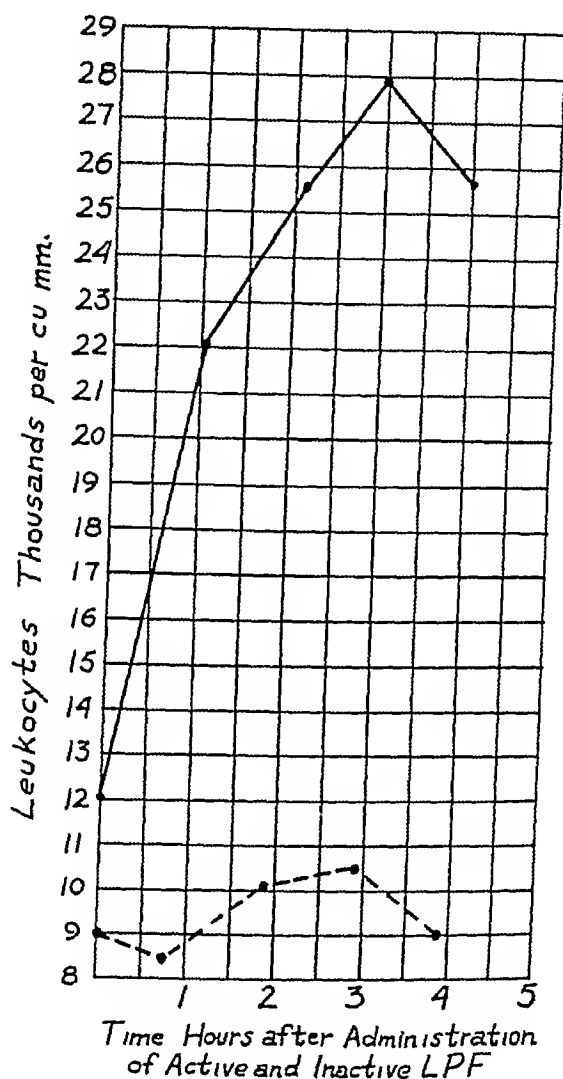


FIG 3 THE INACTIVATION OF THE LPF BY HEAT

Solid line indicates Dog 33-T 25 milligrams of LPF administered. Broken line indicates Dog 30-T 25 milligrams of LPF inactivated by heating at 100°C for 35 minutes.

are insoluble in an aqueous medium. Heating again to 100 C such insoluble material fails to decrease its potency. Its injection in dogs induces a rise of 114.7 per cent in the level of circulating leukocytes. This observation definitely indicates that the LPF can be recovered from aged exudates as a highly thermostable substance. Experiments of this sort appear in figure 4. Such observations would preclude the interpretation that the leukocytosis-promoting factor is a thermolabile substance adsorbed to the pseudoglobulin molecule.\*

\* The insoluble brittle flakes obtained when evaporating the active supernatant to dryness on a steam bath are biuret and ninhydrin positive.

TABLE 4 —Effect of the Soluble Fraction Derived from Aged LPF When Evaporated to Dryness on Steam Bath and also When That Dried Fraction is Boiled for 30-40 Minutes

Dog No	Amount of dried supernatant material derived from aged LPF injected into heart	Basal white cell count	Maximum white cell count following administration of either evaporated material derived from aged LPF or following boiling of such evaporated dried material
	mg	cu mm	cu mm
9-T		7,625	17,500
11-T		11,050	24,200
16-T	10	9,350	20,650
29-T	20	12,950	18,400
Average		10,244	20,188*
9-T†	20	13,500	20,350
11-T†	23	11,575	22,100
39-T†	28	14,750	43,050
Average		13,275	28,500‡

\* Percentage increase in leukocyte level = 97.1%

† The evaporated material to dryness has in addition been subjected to boiling for 30-40 minutes

‡ Percentage increase in leukocyte level = 114.7%

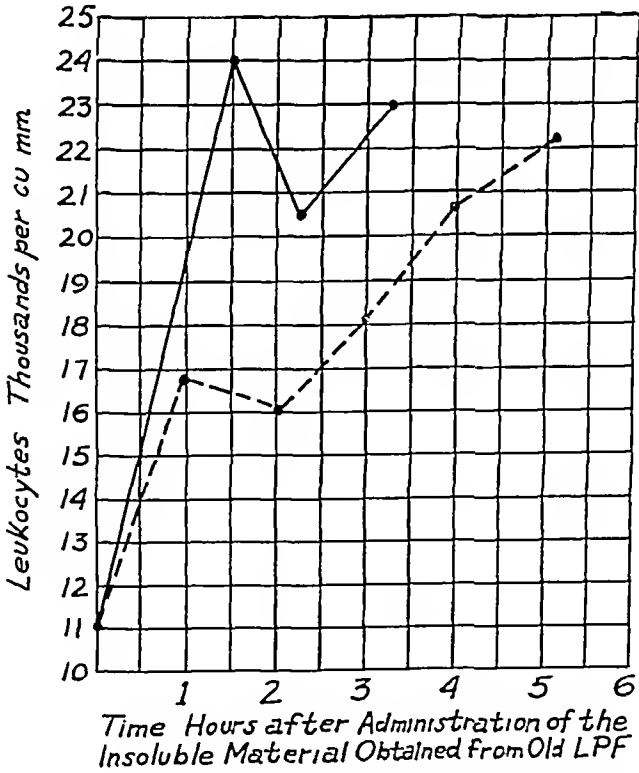


FIG. 4 THE ACTIVITY OF THE EVAPORATED SUPERNATANT FRACTION OF OLD LPF

Solid line indicates Dog 11-T Suspension of supernatant fraction from old sample of LPF, evaporated to dryness and suspended as insoluble material. Broken line indicates Dog 11-T the insoluble material was obtained by evaporating to dryness the supernatant fraction of an old sample of LPF. This was then heated for 30 minutes at 100°C. The procedure essentially failed to inactivate the material.

Polypeptides are known to be highly thermostable.<sup>9</sup> For this reason the amino acid nitrogen before and after hydrolysis was determined on several samples of the active supernatant phase from an aged sample of LPF. These observations are as yet preliminary, but the measurements from such samples indicate in each case a rise in the amino acid nitrogen following acid hydrolysis. The actual figures obtained on such samples before and after hydrolysis are listed in table 5. These

TABLE 5 — *The Amino Nitrogen Content in the Active Principle of the LPF before and after Hydrolysis*

	Before hydrolysis	After hydrolysis
	mg /cc	mg /cc
Colorimetric method (modified O. Folin method <sup>10</sup> )	0.09 0.15 0.16 0.22	0.2 0.37 0.51 1.00
Average	0.155	0.520
Copper method (method of A. A. Albanese and V. Irby <sup>11</sup> )	0.009 0.009 0.009 0.026	0.066 0.053 0.095 0.106
Average	0.013	0.080

observations suggest very strongly that the active principle is a relatively simple polypeptide.

### DISCUSSION

The foregoing observations are consistent with the interpretation that the active principle in exudates, which reasonably explains the mechanism of leukocytosis with inflammation, is a relatively simple polypeptide.\*

Besides the theoretic significance of this fact there is a possibility that the above observations may have practical application. The canine leukocytosis-promoting factor has been shown to be effective in human beings.<sup>5</sup> The active factor has, however, been shown not to be too stable with time. A spontaneous denaturation occurs whereby the material loses its initial solubility. The present observations indicate that in spite of these changes the LPF is split off as a soluble and thermostable component. Its biologic activity remains undamaged. In this way it is conceivable that the leukocytosis-promoting factor of exudates can be preserved for long intervals in spite of age. These facts definitely suggest further practical use of this factor.

### CONCLUSIONS

The leukocytosis-promoting factor of exudates appears to be a relatively simple polypeptide attached to the pseudoglobulin of exudates when extracted from the latter.

\* The active principle is primarily nondiffusible from the active supernatant fraction of an aged sample of LPF. This would tend to indicate that the factor is somewhat larger than an amino acid.

This has been found by a study of samples of leukocytosis-promoting factor which have become insoluble by aging. Nevertheless, the active material has been found to be liberated as a water soluble component which is highly thermostable.

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# THE PLACE OF THE SPLEEN IN THE ENDOCRINE SYSTEM

By ARDREY W. DOWNS, M.D.

FROM being considered as of no use to the organism the spleen has come to occupy a rather insecure position as a gland of internal secretion. The purpose of this article is to try to show how this change has come about. A wide variety of functions has been assigned to the spleen from time to time, most of them involving the life cycle of the blood corpuscles. The spleen has been stated to manufacture red blood corpuscles and, on the other hand, it has been claimed that red corpuscles are destroyed in this organ. In the same way the spleen has been regarded by some as a place where white blood corpuscles are produced and by others as a place where they are destroyed. Evidence of an endocrine function of the spleen is still meagre and to some extent conflicting.

It is necessary to touch briefly on the background. The spleen was known to the ancients, the Greeks considering it as inessential to life. Aristotle refers to it and Erasistratus states that it is useless. It was regarded by the Greeks and Romans as detrimental to a runner and this idea persisted at least to the time of Shakespeare. It is frequently stated that the ancients removed the spleens of runners to increase their speed.

Splenectomy is said to have been performed in Europe in the sixteenth century. Moynihan<sup>1</sup> gives an account of the operation performed by Zaccarelli as written by an Italian physician, Leonardo Fioravanti, for the removal of the spleen from a woman 24 years old. Apparently she made a prompt recovery and suffered no ill effects. Moynihan points out that the suggestion has been made that the mass removed was an ovarian cyst. At least two other removals of the spleen are stated to have taken place during this century. During the seventeenth century two splenectomies are recorded, both of which seem to be reasonably well authenticated. In both cases the reason for the operation was knife wound in the left side with prolapse of the spleen.

The first experiments known to have been performed on animals in a study of the spleen, as described by Moynihan,<sup>2</sup> were carried out by Malpighi in 1669, Clarke in 1676 and Zambecari in 1680. Malpighi ligatured the splenic artery and vein in a dog. Subsequently the spleen underwent complete atrophy and the liver enlarged. Both Clarke and Zambecari performed splenectomies on dogs. No significant change was observed.

In 1841 Bardeleben<sup>3</sup> published the results of the first carefully planned experiments directed toward elucidation of the function of the spleen. The results of complete removal of the organ were briefly these: a transitory decrease in the number of red, and an increase in the number of white, corpuscles in the circulating blood, an increase of activity of the bone marrow and lymphatic glands, no apparent ill effect on life. These experiments are particularly noteworthy because of the care with

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which they were planned, the thoroughness with which they were carried out and the conclusions drawn

It was at this time that general consideration began to be given to removal of the spleen as a therapeutic measure. The results of Bardeleben's experiments gave support to the belief that the spleen was not necessary and splenectomy came to be employed more and more. Collier<sup>1</sup> recounts 29 cases of splenectomy reported to 1882. Of these, 13 were for wandering spleen, enlargement (described as "simple") and hydatid cysts. Eight of these were regarded as successful. In the other 16 cases "leukocythemia" was present. None recovered. These results made it clear that splenectomy was neither a sound nor safe procedure for leukocythemia.

A relationship of the spleen to hematopoiesis was recognized as a result of Bardeleben's experiments, which were confirmed by other investigators. The unsuccessful outcome of extirpation of the spleen in leukocythemia led to a turning of attention to the anemias. At the beginning of the twentieth century any syndrome of splenic enlargement and anemia was classified as a splenic anemia. If the spleen were responsible for destruction of red blood corpuscles then it seemed to follow that a condition such as pernicious anemia should be benefited by removal of the organ. Consequently, during the second decade of the century splenectomy was resorted to in a number of these cases. The results were inconclusive.

During this time Whipple, Hooper and Robschelt had been doing fundamental work on the anemias. Their report<sup>5</sup> on the influence of meat, liver and various extractives on blood regeneration following simple anemia in dogs, produced by bleeding, is the foundation on which was developed our present knowledge of blood formation. From this study came the use of liver in pernicious anemia. Minot and Murphy<sup>6</sup> found that feeding one-half pound of liver daily brought about improvement in patients with pernicious anemia. It later became clear that the fraction of liver which was curative in pernicious anemia was not the one which was effective in the anemia of bled animals. These discoveries led to the abandonment of the surgical procedure of splenectomy as a mode of treatment of anemia.

With the rising interest in internal secretions the spleen was not neglected, though it did not receive as much or so wide-spread attention as some other endocrine glands. During this period among the most notable workers was the group headed by Pearce, with Musser and Krumbhaar and a large number of associates. Among others on this continent who were interested in trying to establish something definite as to the way in which the spleen functions were Leake, Holloway and Blackford, Eddy and Downs. In Europe probably Danilewsky, Stradomsky and Mouzon were particularly interested in the physiology of the spleen.

Investigation of the part that the spleen plays in the formation and in the destruction of blood cells has followed largely four lines. Microscopic examination of the spleen, the counting of the cells in the blood going to and coming from the organ, the results of splenectomy, and the effects of administration of splenic substance or extract.

During a period of about fourteen years centering on the second decade of this century Pearce and his associates carried out many experiments intended to throw light on the function of the spleen. Dogs were the animals used. In 1913 Musser and

Krumbhaar<sup>7</sup> stated that after splenectomy anemia usually develops quickly and reaches its height in from three to six weeks, then the blood picture approaches the normal after about three to four months, with complete return to normal in five to ten months. Accompanying this is marked leukocytosis, which reaches its height in twenty-four hours but persists to a slight degree for several months. In 1912 Karsner and Pearce<sup>8</sup> had reported an increased resistance of red blood corpuscles after splenectomy. This was confirmed the following year by Pearce and Peet<sup>9</sup> who stated further, that the increased resistance cannot be explained on the basis of an increase in reticulated cells in the circulating blood. Practically all observers agree that after splenectomy the red blood corpuscles are less fragile than normally. Pearce, Krumbhaar and Frazier<sup>10</sup> had concluded that the transient anemia following splenectomy is due to the loss of some substance that stimulates the bone marrow with a lack of blood formation, rather than to increased blood destruction. This conclusion was supported by the observation of themselves and others that it was relieved by the administration of splenic extract.

In 1920 Downs and Eddy<sup>11</sup> published results of the subcutaneous injection of single doses of splenic extract in rabbits on the number of red corpuscles in the circulating blood. The immediate effect was a temporary decrease in the number. It was thought that the decrease might be due to a direct hemolytic action of a splenic agent. There was frequently a very transient increase in the number of white corpuscles. In 1921 Eddy<sup>12</sup> enunciated the hypothesis that the spleen produces an internal secretion. This was based on the changes in the erythrocytes after splenectomy, the modification of the blood picture in hyperplasia of the spleen, and the specific effects on the red blood corpuscles of injection of splenic extract. Nothing was known of the chemical nature of the supposed hormone and it was difficult to formulate a consistent theory of its possible mode of action. He suggested that the chief function of the spleen is the removal from the circulation of disintegrated erythrocytes, that the splenic cells elaborate this material and thereby produce an internal secretion, that this internal secretion, possibly after modification by the liver, stimulates the erythrogenic function of the bone marrow and is used up in the manufacture of new corpuscles.

Danilewsky<sup>13</sup> in 1895 was able to cause a marked increase in the number of red corpuscles in the circulating blood and also in the hemoglobin content of the blood by a single intraperitoneal injection of an extract of the spleen. Apparently he was the first to suggest that the spleen acts on the bone marrow. In 1916 Stradomsky<sup>14</sup> had concluded that the immediate effect of the splenic agent was destruction of erythrocytes. The increase in production of red blood corpuscles by the bone marrow, however, went beyond the usual response to a reduction in the number of corpuscles in the circulating blood and he felt that this was explained by assuming the removal of a normal regulating action by a splenic hormone on the bone marrow.

In 1922 and 1923 Downs and Eddy<sup>15</sup> <sup>16</sup> reported further experiments in which splenic extract was administered subcutaneously to rabbits daily for periods of from four weeks to fifteen weeks. These showed the appearance of reticulated cells in the circulating blood in a proportion much greater than normal, the presence of

nucleated red corpuscles in the circulation, and an increase in the resistance of the circulating red blood corpuscles. These results agreed with those obtained previously and appeared to confirm the theory of splenic action that had been proposed.

At this time Leake and Leake<sup>17</sup> demonstrated that both extract of spleen and extract of red bone marrow are hematopoietic agents and that a combination of the two is more powerful than either one alone. In their opinion they act first by increasing the rate of production and second, by causing an extension of functioning red marrow. Leake and Evans<sup>18</sup> followed the treatment of various types of anemia in humans by the use of desiccated spleen and red bone marrow combined in equal quantities. Improvement was obtained in grave secondary anemias, both active and chronic, in dietary anemias of infants and in menorrhagic anemias.

The development of the concept of the spleen as a gland of internal secretion has taken place gradually during the past quarter century. During later years it has been based almost entirely on pathologic and clinical observations. In 1916 Kaznelson<sup>19</sup> showed that removal of the spleen was followed by a rise in the platelet count and clinical improvement in some cases of thrombocytopenic purpura. He concluded that the spleen had been exerting an excessive cytolytic action on platelets. Whether Kaznelson's conclusion was sound or not his recommendation that splenectomy be performed in cases of thrombocytopenic purpura was followed by the report of excellent results in 16 cases.<sup>20</sup> Troland and Lee<sup>21</sup> in 1938 described a substance obtained from spleens that had been removed from patients suffering from thrombocytopenic purpura which caused a reduction in the platelet count when injected into animals. Wiseman and Doan<sup>22</sup> in 1942 described a condition that they ascribed to hyperactivity of the spleen. The spleen was enlarged and the white blood corpuscle count low. They named it primary splenic neutropenia. Splenectomy was followed by an increase in the leukocyte count and improvement in the condition of the patient. They believe excessive activity of a splenic hormone to be the cause, without being able to determine what has led to this disturbance of splenic processes. In 1946, Doan and Wright<sup>23</sup> described primary splenic panhematopenia, in which there is a reduction of all formed elements of the blood, red corpuscles, white corpuscles and platelets. In these cases also splenectomy caused marked improvement.

This study of the spleen is not unmindful of the useful purpose served by the spleen as a reservoir of quickly available red blood corpuscles with their hemoglobin. Nor does it overlook the phagocytic activity of the gland. It is, however, concerned with a different problem. Moreover, it has been pointed out that the phagocytic action and the production of one or more agents affecting the red blood corpuscles in active circulation and the bone marrow may be closely related.

#### CONCLUSION

An attempt has been made to trace the scientific study of the function of the spleen from the time of ancient Greece to the present. Modern theory and experimentation have linked the organ with the formed elements of the blood. Gradually a theory of endocrine activity has been developed which relates the spleen to the corpuscle content of the circulating blood. A normal blood picture is due, in part at



least, to a normally functioning spleen. There is then the possibility of hyper- or hypoactivity and it seems reasonable to regard certain clinical entities as due either partly or wholly to disordered splenic activity. Those conditions in which it seems to be fairly well established that removal of the spleen should be considered are thrombocytopenic purpura, splenic neutropenia and primary splenic panhematopenia. Cases so far reported suggest strongly that the prognosis is much improved by splenectomy. What the later effect of absence of the spleen may be remains unknown. A clinical manifestation of hypoactivity of the spleen does not appear to have been recognized as yet.

### SUMMARY

While the evidence for an endocrine function of the spleen is meagre and the exact nature of this action is not clear it does seem to be fairly well established that the spleen must be considered as an important part of the mechanism whereby a normal corpuscle, and possibly platelet, content of the circulating blood is maintained.

This regulation seems to be due to the production by the spleen of one or more hormones.

These hormones appear to affect the processes of the bone marrow and also may act upon the corpuscles in the circulating blood.

The conception of hyperactivity of the spleen in the human being seems to be firmly established but little is known of the clinical appearance of its hypoactivity.

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# A HEMATOPOIETIC PERIFOLLICULAR ENVELOPE IN THE RAT SPLEEN

By E B KRUMBHAR, M D , Ph D

IT IS well known that hematopoietic cells of various kinds occur normally in the spleen of rats and other rodents as well as of other animals. In addition to these lymphocytes and reticulo-endothelial cells found in the Malpighian follicles and accompanying small arterioles, these cells may also be found in the pulp, together with erythrocytes and occasionally neutrophils, eosinophils, plasma cells, megakaryocytes, myelocytes, and nucleated erythrocytes (Jaffé<sup>1</sup>). These cells are usually described as occurring diffusely or in small clumps. It has apparently escaped general notice, however, that surrounding the Malpighian follicle is an envelope of hematopoietic cells which is fairly well circumscribed, lacks sinuses, is separated from the follicular cells by a shell or rind of connective tissue, and responds to hematopoietic stimuli differently than does the red pulp. Under certain pathologic conditions, it may appear to be more voluminous than the follicle, which it surrounds. To be sure, Jaffé speaks of a *Follikelhof* or *Aussenzone* from which the follicle is sharply separated, which Weidenreich<sup>4</sup> called a *Randzone* and Strasser<sup>2</sup> a *Follikdaussenzone*. Strasser also noted absence of sinuses and a thin rind. Also, in a study of the toxicity of benzene in rats and other animals, Svrbely, Dunn and Von Oettingen<sup>3</sup> noted "narrowing of the perifollicular collars of closely packed pale cells." Dr R D Lillie, in whose laboratory this work was performed, writes that he "conceives of this zone as possibly the pressure reducing mesh homologous to the ellipsoids of dogs and cats. Its conspicuousness is quite variable. To me [R D L] it has seemed to vary inversely with the blood content of the pulp and sinuses. It is often the principal site of hemosiderin accumulation." As will appear below, we too have found the area to vary greatly in size and content under different conditions and in general to have its size modified by the amount of blood in the red pulp. However, under our conditions, at least, it seems to be rather an auxiliary hematopoietic tissue, depleted when exposed to hematopoietic poisons, and exuberant during periods of active blood cell regeneration.

## PRESENT STUDY

The preparations studied were mostly routine paraffin cut sections (6-8 micra in thickness) stained with hematoxylin and eosin. Other stains, such as eosin-methylene blue and azur 2-eosin were also used. Details of the records of the experiments tried, leukocyte and differential counts, and the weight and gross appearance of the spleen were of considerable help in evaluating the findings. During the examination of the hematopoietic system of several hundred rodents, chiefly adult rats, during the course of a study of the pathologic effects of mustard gas and other hematopoietic poisons, it soon became apparent that the spleen contained two rather distinct hematopoietic areas in addition to the lymphocyte-forming Malpighian

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follicles. These were (1) small foci scattered through the red pulp, (2) the perifollicular areas already referred to. The small foci were apparently composed of either myelocytic, erythrocytic, monocytic, or lymphocytic cells, or sometimes of mixtures. In the case of the lymphocytes, they may have been about small vessels, which, as Jaffe notes, can be brought out with the perfused specimens, the cells of some of these foci have the same appearance as those in the envelopes about to be described. As might be expected, these foci tended to disappear in the spleens depleted by poison, and to be large and widespread in recovery periods during active

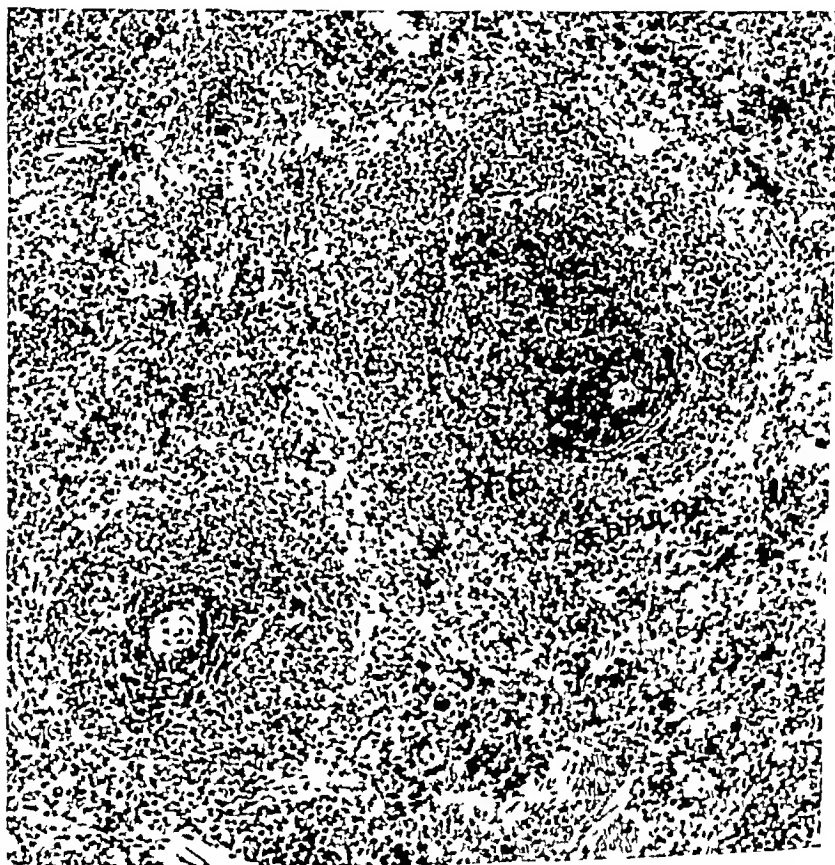


FIG. 1. Normal rat's spleen. Two Malpighian follicles (MF) with their central arteries appear, surrounded by the paler staining perifollicular envelope (PFE). The more open red pulp occupies the rest of the field (H-E, 150 X).

regeneration. However, as so often is the case with different units of the hemopoietic system in the present state of our knowledge, they at times showed unexpected and unexplained responses.

In sections of normal rats' spleens, the perifollicular areas are easily distinguished in the stained specimen as collars of nucleated cells, paler and larger than the adult lymphocytes of the Malpighian follicle. The "collar," a term appropriate for the two dimensional section but not for what is obviously a three dimensional structure, varies in width from about 30 to as much as 200 micra. It is separated from the follicle by a delicate but definite line of collagenous connective tissue which is

presumably a development from the follicular framework. This narrow rind contains a young type of fibroblast and sparse collagen fibers. The rind usually cannot be traced around the complete circumference. In one instance (rat 1630), a capillary was enclosed in this band for about one quarter of the perimeter. The cells of the perifollicular areas in normal spleens are mostly mononucleated, about the size of young lymphoblasts, the nuclei round and moderately full of chromatin, often with a deep staining central dot, and with scanty pale-staining cytoplasm. They show

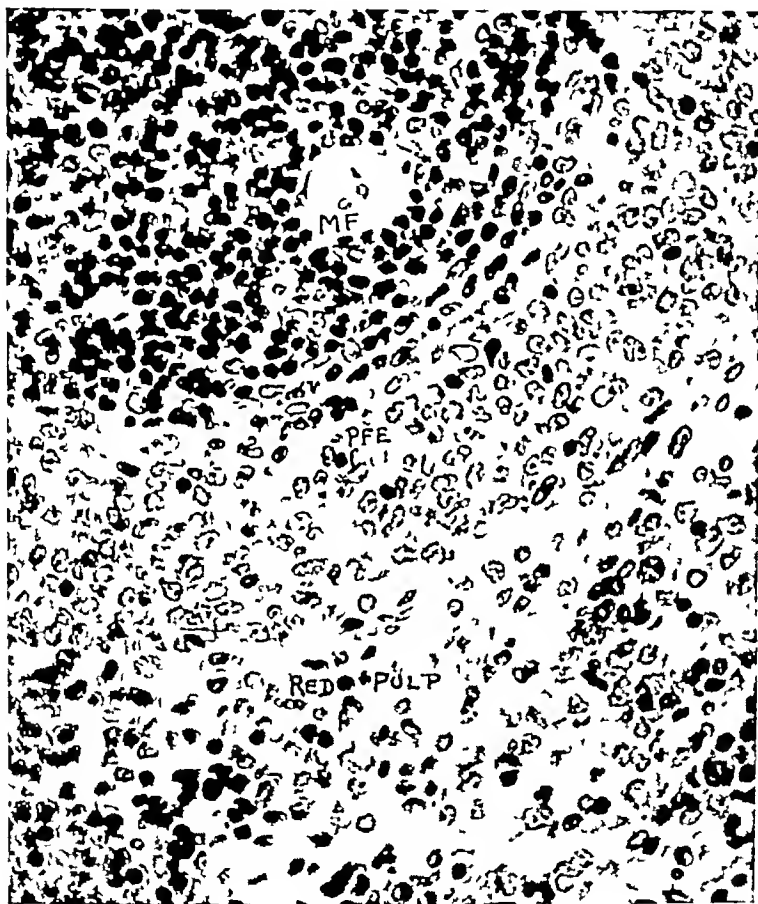


FIG. 2. Same section, showing part of the larger follicle and its collar. Note the curved rows of adult lymphocytes near the margin, separated by delicate connective tissue (which does not show in the photograph). The more homogeneous nature of the collar than of the red pulp is obvious (H-E, 320 X).

no gradation to the fibroblasts of the thin rind, nor do they resemble them in appearance.

They do not appear to be monocytes, as they have scanty cytoplasm that is not granular and never contains ingested material. The nuclei are never indented and show no characteristic nucleoli.

These cells were further investigated by making imprints of the freshly bisected surface of some 30 normal rats' spleens and comparing the stained imprint (May-Grünwald-Giemsa) with stained sections from the other side of the bisection. Successive imprints, a dozen or more per spleen, reproduced the same appearance.

with surprising similarity, given a marked, if superficial, resemblance to serial sections. Though the blood cells did not stain as well by this method as they do in good blood smears, the cells in question were easily identified. They were thought to be young lymphocytes by several colleagues as well as by myself. Compared to the small dark-staining nuclei of adult lymphocytes and normoblasts, in the imprints their nuclei were 50 per cent larger, paler, with coarse chromatin clumps and a tendency to condense around the nuclear membrane. The cytoplasm was mostly very scanty, where more abundant, it stained a very pale blue without granules or

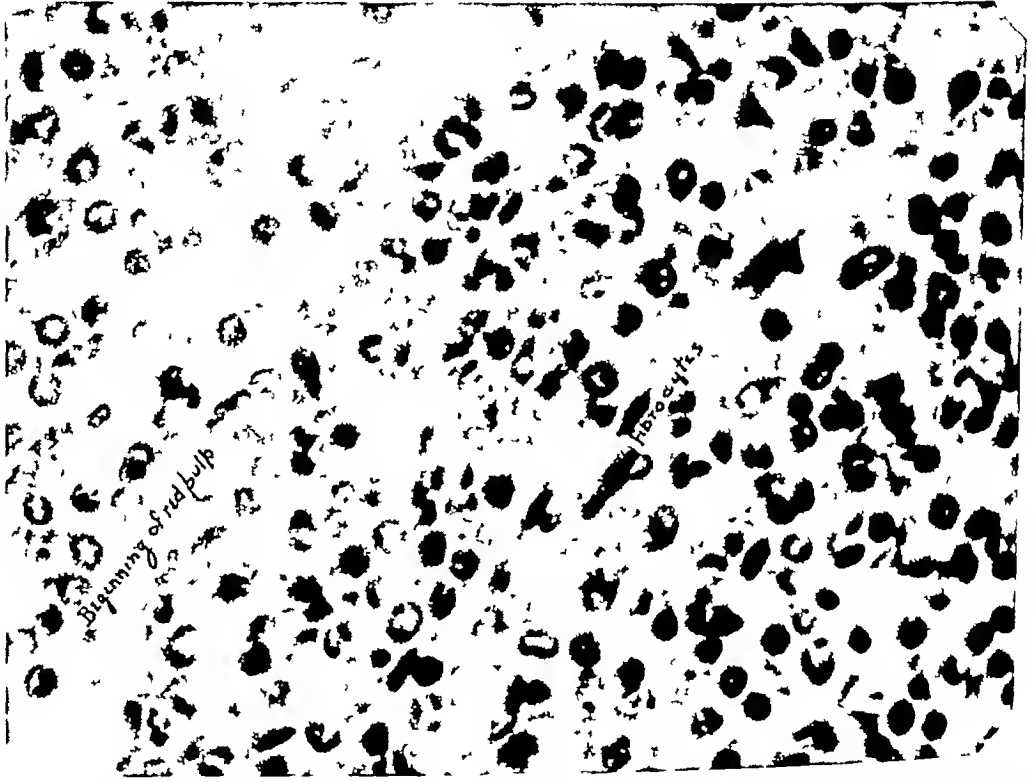


FIG 3 Greatly depleted spleen of rat 6-2-7, dying seventeen days after nitrogen mustard gas poisoning. Both the follicle and collar have lost many cells, with a number of polys, immature red cells and debris present. Note the fibrocytes separating follicle and collar (H-E, 135 X)

vacuoles. Oxidase granules (benzidine stain) could not be demonstrated in their cytoplasm such as appeared in the neutrophils.

The possibility that these cells are immature monocytes or myeloid cells cannot be ruled out until studies can be made under living conditions, as in tissue cultures, and such items as their mode of growth and locomotion observed. Occasional larger round, heavily stained nuclei (immature cells) are found, and more sparsely and not constantly, normoblasts and adult erythrocytes, polymorphonuclear neutrophils and eosinophils, also large, pale vesiculated nuclei that are taken to be adult reticulo-endothelial cells. No megakaryocytes have ever been found in the collar, and no sinuses. The outer margin of the zone merges almost imperceptibly into the red pulp, the transition being chiefly determined by the appearance of

sinuses and more stroma and by the much greater number of erythrocytes. Sections that cut through the edge of a follicle may show a broader perifollicular than follicular area, or even none of the adult lymphocytes of the true follicle at all.

In the spleens depleted by various poisons of the lymphocytic and granulocytic cell series, the perifollicular collar may be represented by an almost empty circular band (except for an extremely scanty stroma) bounded on its two margins by a still recognizable follicular rind and the denser stroma of the red pulp. If the damaged spleen is congested, the empty collar stands out in bold contrast.

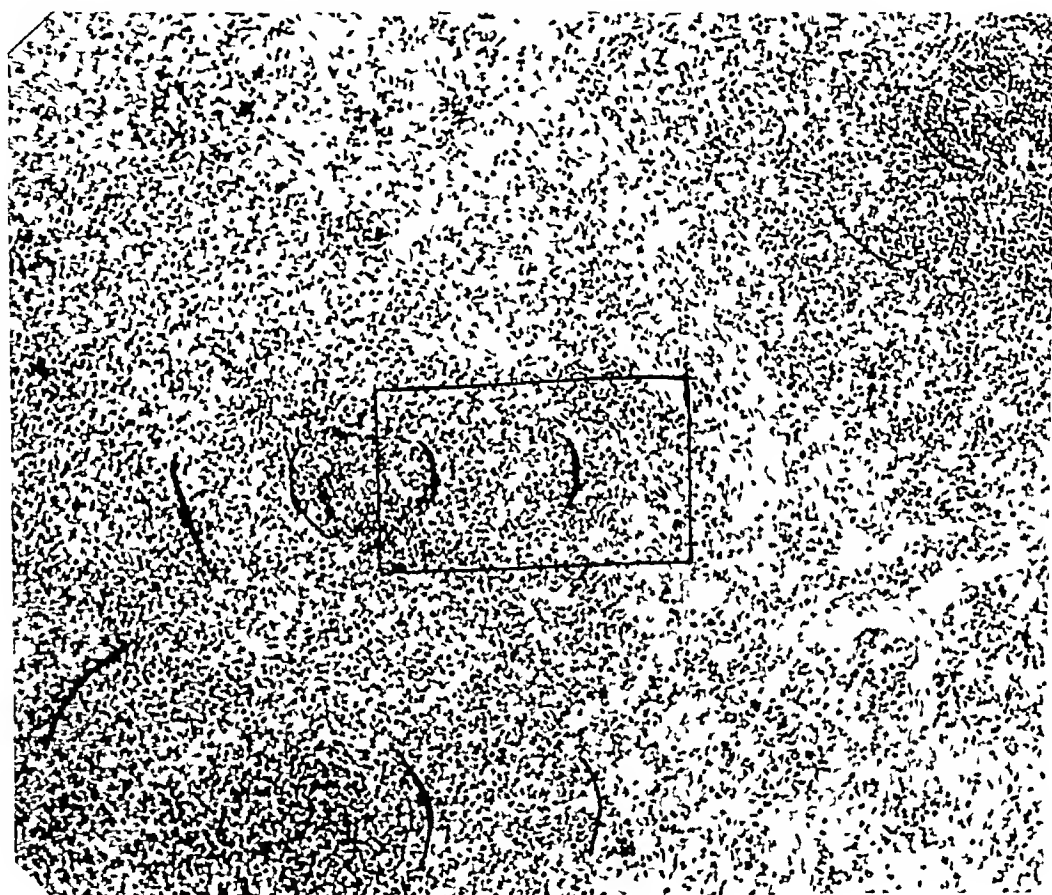


FIG. 4. Spleen of rat 6-1-3, killed while recovering from a non-lethal dose given three weeks earlier. Note the extremely wide collar of a follicle that is relatively small. The rectangle indicates the field of the next figure with an approximation of the width of some collars indicated. (H-E, 150 $\times$ )

In spleens of rats killed three weeks to three months after the experiments, the collars usually but not always appear larger and paler than normal with larger cells and occasional mitoses present. More hematopoietic cells of several kinds, except the megakaryocyte, are found interspersed and sometimes in concentrated foci that suggest local production. As the collars contain only few erythrocytes, the more the red pulp is congested, the more conspicuous the relatively pale, reddish purple, collars appear between the dark blue of the follicle and the red of the erythrocytes. In rare instances, however, hematocytic regeneration in the pulp foci may be so

extensive that more erythrocytes are found diffused through the collars together with their usual cells than in the pulp, which is hardly recognizable as splenic tissue

Turning to other rodents—in the mouse spleen, the collar is less developed, nucleated cells are much more frequent throughout the pulp and the transition from follicle more gradual. The "collar" therefore is almost indistinguishable, though occasional suggestions of a connective tissue rind may be seen, beyond which the lymphocytes are more condensed than farther out in the pulp. Here, too, the numerous megakaryocytes in the pulp aid in the differentiation

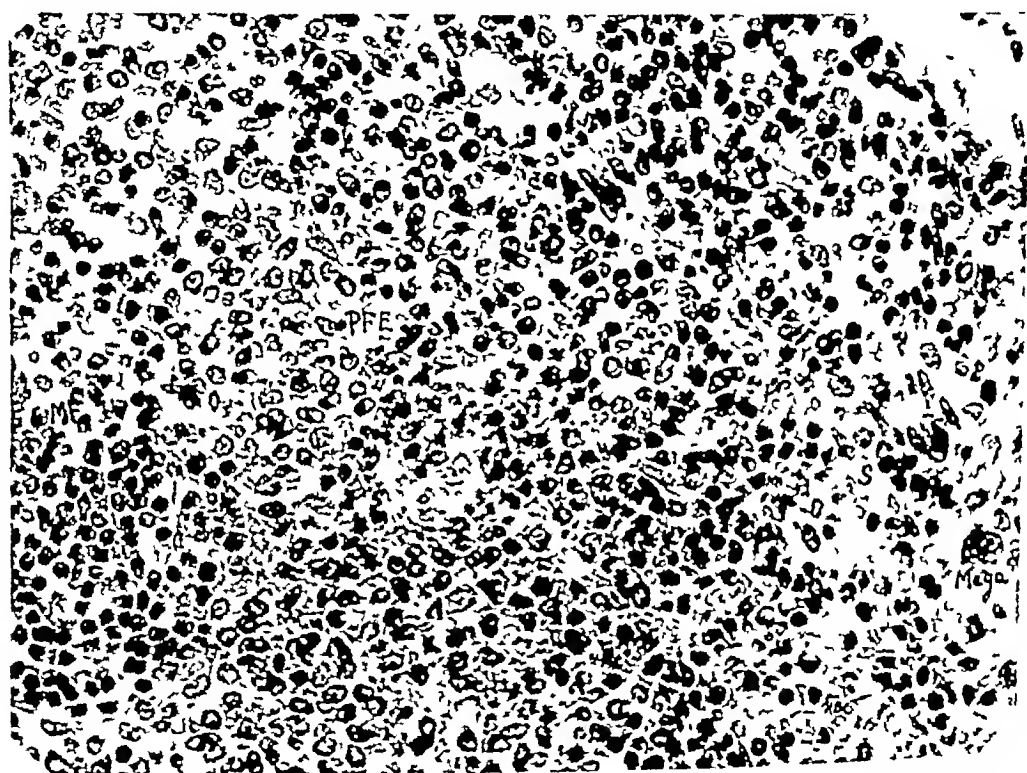


FIG. 5. Same section (800 X). The beginning of the red pulp area is hard to distinguish, though the megakaryocyte (mega), sinuses (s), and RBC (to the right) are surely in the red pulp (H-E)

In the rabbit, the picture is more like that of the rat, except that "pale centers" (reticulo-endothelial cells<sup>2</sup>) may often be found in the topographic center of the follicle, they are rare in the rat. The rind of connective tissue is usually found, the limits of the follicle being indicated both by this pink staining strip and by a tendency for the sectioned tissue to separate at this place. The cell population of the collar varies as in the rat, with a fair number of nonlymphocytic cells that are not to be found inside the rind.

In the guinea pig, which may have pale centers in the follicles, both rind and collar are scanty and ill-defined, indeed, even their existence in places seems doubtful, though Jaffé says the collar is easy to find if perhaps narrow. When found, it has the same difference in cell population from the follicle as does that of the rat. In a few normal hamsters, the perifollicular tissue is also found to be less constant

and rich than in rats. Few if any myeloid cells are seen, ripe or unripe, and very few normoblasts and erythrocytes. The rind varies much in size, not being found at all in small follicles.

### SUMMARY AND CONCLUSIONS

About the Malpighian follicles of several species of rodents, and especially prominent in rats, is a perifollicular envelope composed of hematopoietic cells that takes active part in hemolytopoietic changes.

The identity of the mononuclear cell comprising the greater part of this tissue has not been positively determined, it is probably a young lymphocyte. It is possibly the homologue of the pale centers of the Malpighian follicles in man and other mammals, though it has been found in rabbit spleens which also have pale centers. It should be possible to determine this identity by the use of a greater variety of stains, and of test poisons, and, if possible, of dynamic methods such as tissue culture and moving pictures.

The envelope is separated from the Malpighian follicle by a thin rind of collagenous connective tissue, but on its outer margin it merges gradually with the red pulp. It often contains a scattering of erythrocytes, normoblasts, polymorphonuclear neutrophils, and rarely eosinophils and pigment-bearing macrophages. Some of these cells were so greatly increased under the pathologic conditions first studied that colonization was suggested, they were later thought to have probably wandered in. The collar never contains megakaryocytes or sinuses or blood vessels of any noteworthy size.

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# Case Report

## MONOCYTIC LEUKEMIA

By CARL J. GESSLER, M.D.

THE publication of detailed case reports may often be of value. A case of monocytic leukemia is reported here in detail, in the hope that it may throw some light on our knowledge of this condition.

### REPORT OF CASE

The patient was a 36 year old, married white woman, who lived in Brussels. When first seen on August 8, 1946, her chief complaint was that of progressive weakness of several months' duration. The most interesting feature of the past history was a series of skin symptoms. In January, 1946, an itching erythema appeared on the inner aspect of both thighs, and, one month later, the forearms and neck became similarly affected. These manifestations disappeared about the middle of March. The patient stated that for about eight days at the beginning of July she had had some kind of eczema on the neck. A few days later, on July 18, numerous brownish-red, slightly itching papules had appeared on the trunk, and had persisted for about a week.

The patient was very pale, and her face was swollen. The spleen was not palpable, no lymph glands were felt, the liver was tender and felt 2 cm. below the costal margin in the mid-clavicular line. Blood laboratory examinations revealed the following: Hemoglobin 28 per cent = 4.37 Gm. per 100 cc., red blood cell count, 222 million per cu. mm., color index 0.6, total white blood cell count 50,500 per cu. mm. Examination of the stained smear (May-Grünwald, Giemsa) revealed the presence of numerous large cells with irregular nuclei. The differential count (based on 400 white cells) was as follows:

- 20.25% neutrophils (9.25% of them staff cells)
  - 0% eosinophil
  - 0% basophil
- 12.75% lymphocytes
- 56% monocytic cells of abnormal morphology      65.5 leukemic cells
- 9.5% paramyeloblasts
- 0.5% neutrophil metamyelocytes
- 1% neutrophil myelocytes
- 3% normoblasts were found per 400 leukocytes

Aspiration of the bone marrow by sternal puncture proved unusually difficult, finally, however, small lumps of whitish material were obtained, practically free from blood. The smear of the specimen was almost entirely made up of leukemic cells, which could be classified as pathologic promyelocytes or promyelocytoid paramyeloblasts. *Not one cell was seen in the sternal puncture preparation which was comparable with the monocytic type of cells found in the blood.*

The patient was seen again three weeks later and at that time her condition had deteriorated rapidly. Considerable edema of the face, extreme weakness and extensive ulcero-necrotic lesions of the mouth were noticed. The temperature was 39°C (102.2°F). A few brown macules, half a centimeter in diameter, remained from an extensive eruption of red spots which had appeared during the interval between the two examinations. The blood pressure was 120-40. Blood examination showed the following: Hemoglobin 11 per cent = 1.72 Gm. per 100 cc., red blood cells 1.04 million per cu. mm., and leukocytes 184,600 per cu. mm. The differential count was as follows:

- 3.25% neutrophils (1 staff cell)
  - 0% eosinophil
  - 0% basophil
- 4.25% lymphocytes
- 77.5% monocytic cells of abnormal morphology      92 leukemic cells

14.5 % paramyeloblasts

0.5 % plasmacyte

2 normoblasts were found per 400 leukocytes

The patient died on August 31

### DISCUSSION

The ulcero-necrotic lesions of the mouth were perhaps particularly significant. Forkner<sup>1</sup> stated that such lesions are more constant and extensive in monocytic leukemia than in other varieties of leukemia. The cutaneous manifestations are also worthy of note. Some of them were atypical and probably allergic in origin,

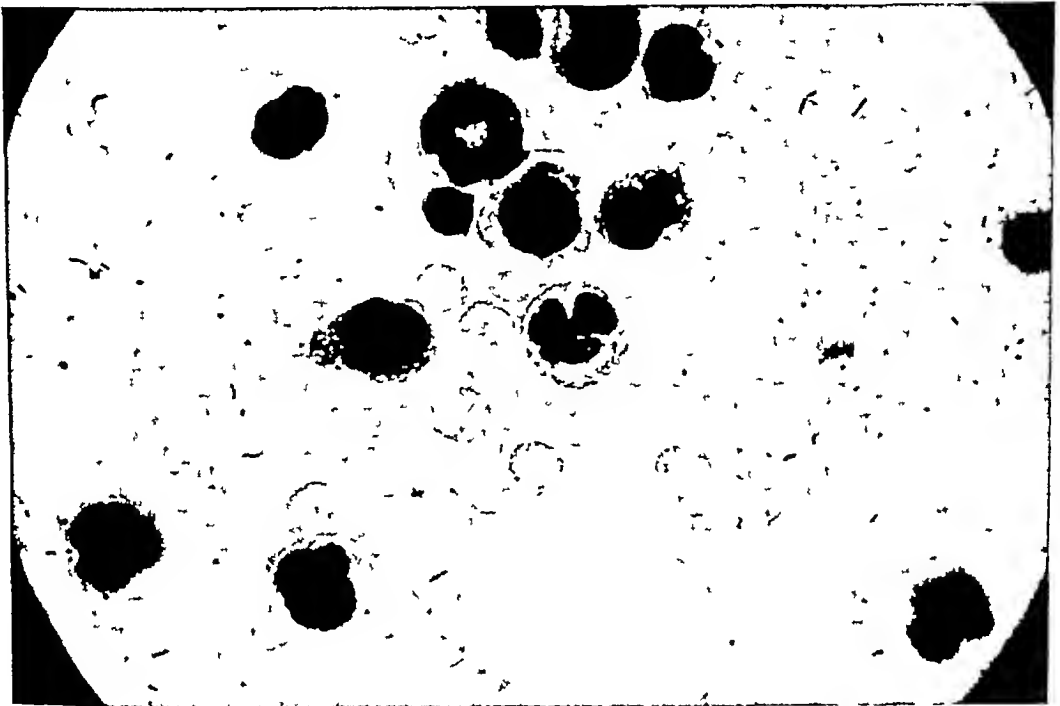


FIG. 1. BLOOD SMEAR

while others, consisting of brown or red maculo-papulae, were more or less characteristic of the reticulo-endothelioses.

There seems to be no possible doubt as to the diagnosis of leukemia. However, opinions could differ as to the exact classification. A diagnosis of monocytic leukemia was substantiated by the high percentage of abnormal monocytic cells in the circulating blood. These cells are extremely polymorphous and can be divided into two main groups, although such divisions are always somewhat arbitrary. (1) The majority were large cells containing irregular nuclei without nucleoli, the pale blue protoplasm being entirely filled with a great number of very small azurophil granules, which are characteristic of the monocyte. (2) Other cells, very similar to the previous ones, had younger nuclei, containing nucleoli, and the fine azurophil granulation occupied only part of the protoplasm, sometimes being confined to the perinuclear zone. It is on the basis of these criteria that Osgood<sup>2</sup> distinguishes monocytes and promonocytes. In the first differential count, in a

total of 56 monocytic cells, 50 belong to the first group and 6 to the second, in the second differential count, on a total of 775 monocytic cells, 465 belong to the

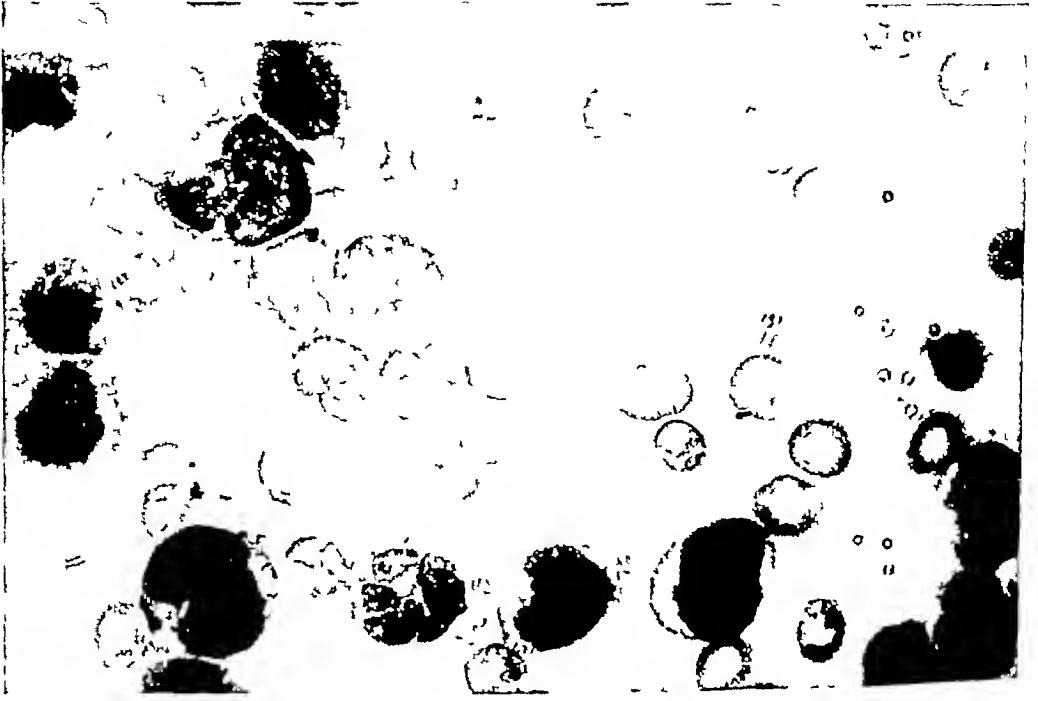


FIG 2 BLOOD SMEAR

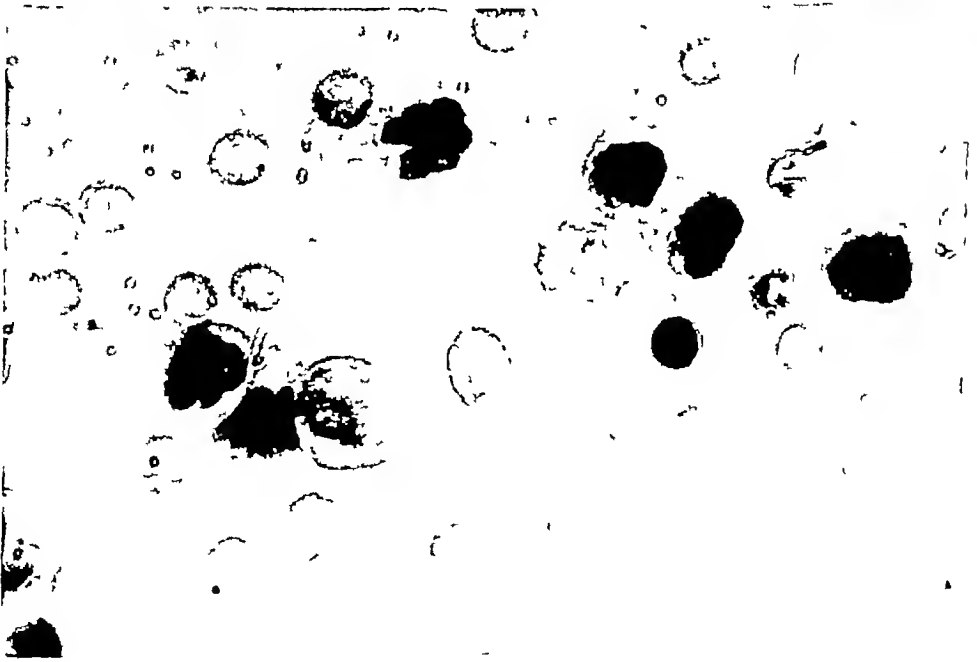


FIG 3 BLOOD SMEAR

first group and 31 to the second, which shows an increase of the more immature cells

However, as mentioned before, there was a marked discrepancy between the

blood smear and the marrow smear. Considering only the marrow smears one would have no hesitation in making a diagnosis of myeloid leukemia. The case could then be interpreted in two different ways. (1) If one believes that the monocytic cells of the blood are derived from the leukemic cells in the marrow, the case could be



FIG 4 BLOOD SMEAR



FIG 5 MONOCYTIC CELL WITH PSEUDPODE  
(Note granules extending within the pseudopode)

termed *paramyeloblastic leukemia*. (2) When one assumes the monocytic cells of the blood are *not* derived from the leukemic cells in the marrow, but express a reaction of the reticulo-endothelial system elsewhere in a patient with myeloid leukemia which Oberling<sup>3</sup> has called "reticulosés associés." Oberling has given instances of this association.

In our opinion, it would be an overextension of the concept of the "paramyelo blast" to consider, as such, the monocytic cells in the peripheral blood of our case

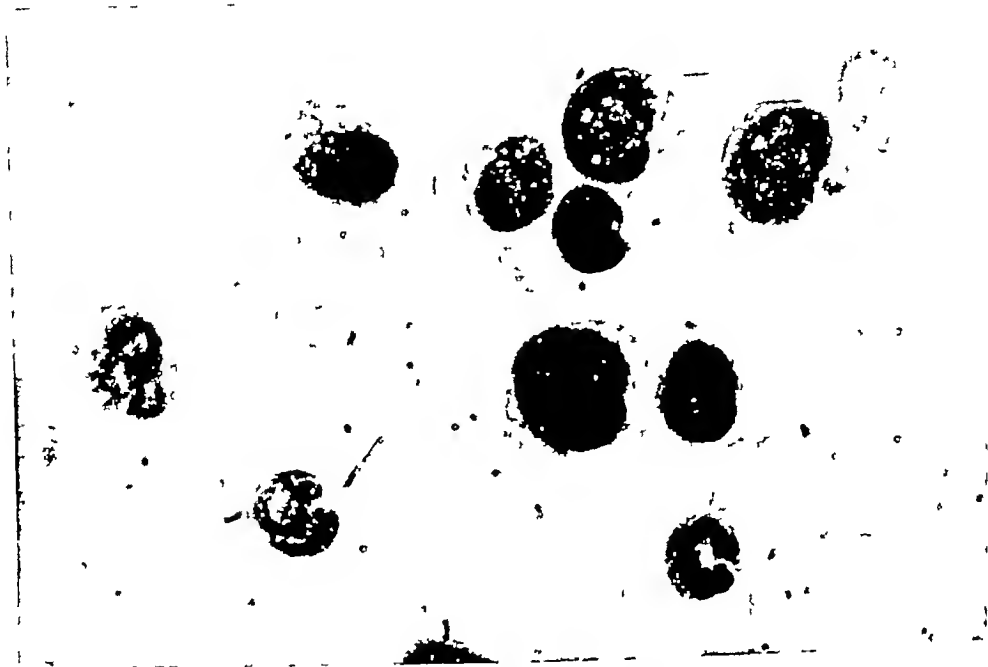


FIG 6 MARROW SMEAR

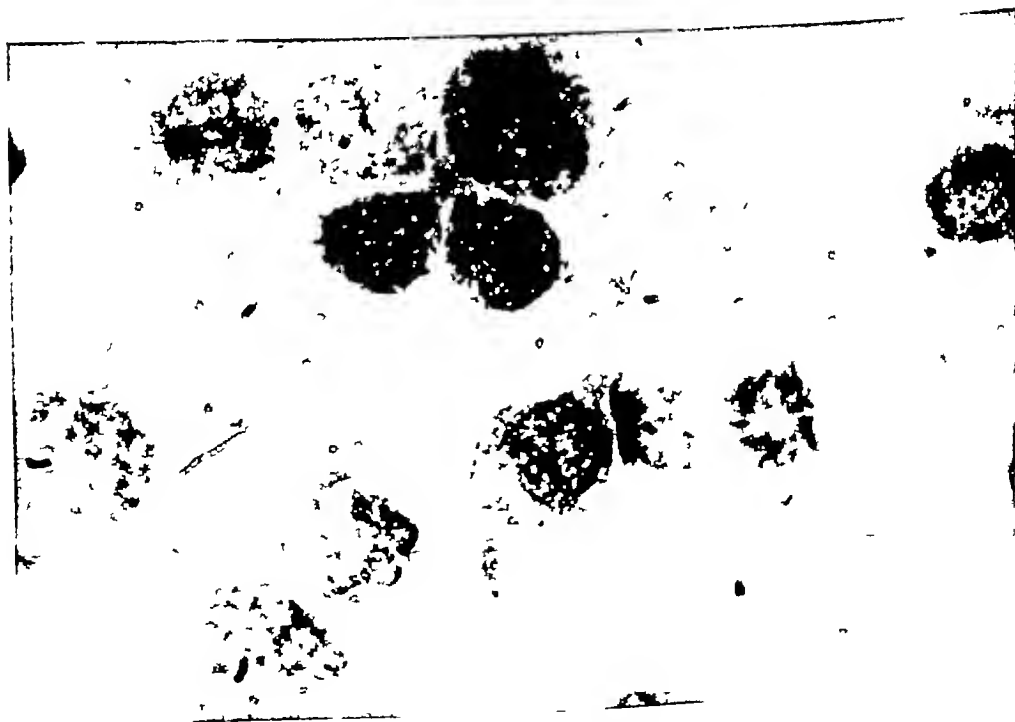


FIG 7 MARROW SMEAR

On the other hand, their morphology seems too definitely abnormal to regard them simply as a result of a simple reaction of the reticulo-endothelial system. Despite the lack of monocytes in the bone marrow, it was felt that there was strong evi-

dence for a diagnosis of monocytic leukemia, which can be defined further, according to the currently prevailing classification, as belonging to the Naegeli type (characterized by leukemic proliferation in the bone marrow) as opposed to the Schilling type (or leukemic reticulosis) characterized by proliferation of the reticulo endothelial system elsewhere than in the bone marrow

*Author's note*

It is interesting that the patient's brother died in 1942 at about the same age (35) of a mediastinal tumor. Unfortunately, no answer was received to our inquiries concerning the nature of this tumor, which might well have been a localized growth of reticulum cells

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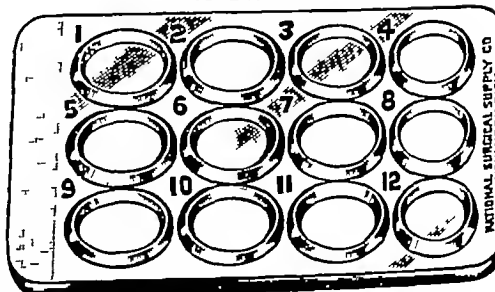
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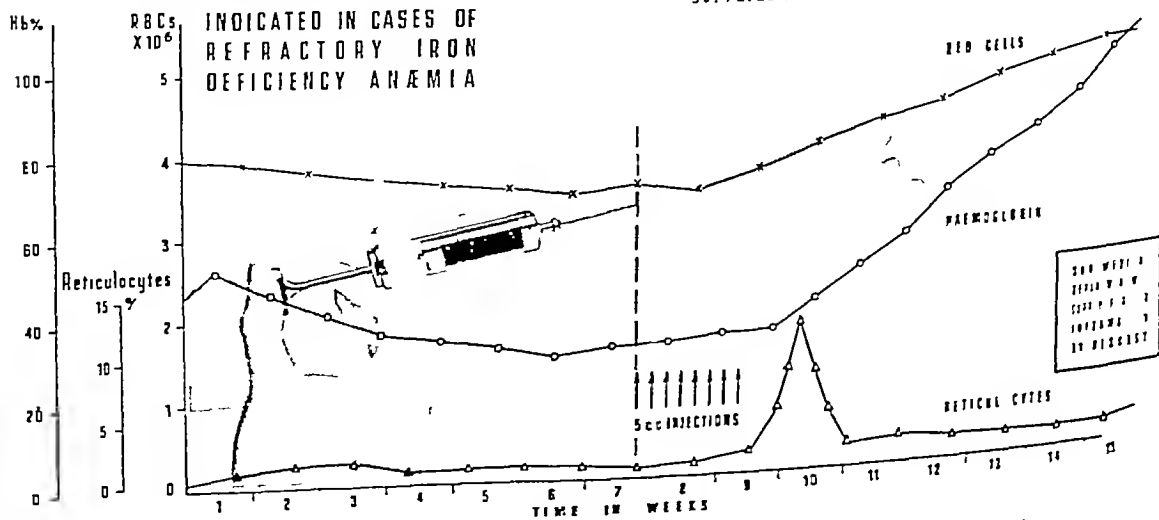
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# BLOOD

## *The Journal of Hematology*

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### NUCLEAR STRUCTURE VERSUS NUCLEAR PATTERN\*

By OLIVER P. JONES, PH D

#### INTRODUCTION

IN A RECENT study of primitive erythroblasts,<sup>1</sup> it was shown that cytoplasmic areas originally interpreted as hyaloplasm by hematologists actually represent negative images of underlying cell organoids—mitochondria and Golgi elements. This finding immediately unfolded many interesting problems concerning erythroblasts in general, especially since it was shown that cell organoids may be preserved in good dry smears. How long do mitochondria retain their vitamin A content? Do the more mature erythroblasts contain mitochondria which have lost their affinity for acid fuchsin and supravital dyes? How long do mitochondria persist in non-nucleated corpuscles of the primitive and definitive generations?<sup>2, 3</sup> What happens to the chromophilic and chromophobic portions of the Golgi element as primitive erythroblasts mature?<sup>4</sup> Do these elements contain vitamin C?<sup>5</sup> How do these organoids respond under the influence of antipernicious anemia substances?

In order to investigate some of these problems various preparations were made from the 15 day rat embryonic liver. A study of lymphoid cells (i.e., any cell with basophilic, nongranular cytoplasm, regardless of its nuclear detail) by means of phase microscopy revealed the presence of more cell organoids, particularly mitochondria, in definitive erythroblasts than had been observed in primitive erythroblasts. Furthermore, most of these organoids were contained in the thin layers of cytoplasm above and below nuclei as seen in dry smears. Although it had been observed previously that some sudanophilic material may be distributed in these regions in primitive erythroblasts from 11 day rat embryos,<sup>1</sup> the significance of this finding was not appreciated fully until rat and human fetal liver material were studied. Since the implications derived from these observations might markedly alter present day concepts in practical and theoretic morphologic hematology,<sup>6</sup> it was deemed necessary to forego the other problems and to undertake the present one dealing with nuclei as seen in dry smears.

#### MATERIAL AND METHODS

Most of the material used for the present investigation was obtained from 136 livers of 15 day rat embryos from 13 pregnant Wistar rats, blood from 11 and 15 day

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embryonic rat yolk sacs, and lymph nodes from adult rats. Human material was obtained from the livers of 4 fetuses ranging 3 to  $4\frac{1}{2}$  months old. \* Dry smears of embryonic livers and lymph nodes from adult rats were made by touching their cut surfaces to a cover slip, smearing them slightly and then drying rapidly. Some of these were stained with the May-Grünwald-Giemsa combination of Pappenheim, using buffered distilled water for dilution. Others were mounted, unstained, in a drop of 10 per cent formalin (4 per cent formaldehyde), and ringed with vaseline for study by phase microscopy.<sup>1</sup> Some of these preparations have remained in good condition for 12-14 months. Moist smears following Zenker-formol fixation were mounted unstained in glychrogel for study by phase microscopy. In order to study both sides of stained blood cells, small square cover slip preparations were mounted in clarite on  $24 \times 60$  mm cover slips. Supravital preparations were made by immersing the yolk sacs and embryonic livers in saline solutions of neutral red (1:10,000), dahlia violet (1:20,000) and janus green (1:20,000 and 1:40,000). The first two were also used in combination according to Baker.<sup>4</sup> After the material had been in these solutions for fifteen to twenty minutes at room temperature, it was swished in a drop of the same solution on a clean slide in order to free some blood cells, cover slipped and sealed with vaseline. Small pieces of yolk sac were also flattened and mounted. Permanent mitochondrial preparations were made in a manner slightly different from that reported previously. Dry smears were fixed in a formol-Muller's fluid (1:9) for two to three hours, washed briefly in distilled water and placed in 1 per cent osmic acid for one-half to one hour in the dark. They were washed and stained with aniline acid fuchsin as described before.<sup>1</sup> The distribution and localization of lipoid material was studied with the aid of Sudan black.<sup>1</sup> The presence of vitamin A was detected by using the Carr-Price reagent.<sup>1</sup>

Phase microscopy has been a great aid in studying the cytoplasm of unstained cells,<sup>1-6</sup> as well as in making optical sections through nuclei. Four diffraction plates were used as follows: 0.2A-0.25 $\lambda$  for a preliminary survey of material, 0.15A-0.25 $\lambda$  for detailed examinations and camera lucida work, 1.0B-0.2 $\lambda$  in conjunction with supravital preparations, and 2.5B-0.33 $\lambda$  alone or in conjunction with Sudan black preparations. The first diffraction plate produced bright contrast whereas the last three produced dark contrast.<sup>7-8</sup> A Spencer research microscope equipped with apochromatic objectives and 15X compensating oculars was used.

## OBSERVATIONS

### A. Blood From 15 Day Rat Embryos

Although this paper deals chiefly with observations made on lymphoid cells in embryonic liver, it seems advisable to report results of certain preliminary studies on blood from the 15 day rat embryo. This material was obtained by pipetting blood from the heart or vitelline vessels.

1. *May-Grünwald-Giemsa Preparations.* Over two thirds of the cells were nucleated and about 85 per cent of these belonged to the primitive erythroblastic series or pre-hepatic generation. The cytoplasm usually stained polychromatophil and it

\*Kindly furnished by the Department of Obstetrics and Gynecology, Buffalo General Hospital

contained some juxtanuclear light areas not unlike these referred to as the area of the cytocentrum. The nuclei were in various stages of pyknosis.

2 *Formol-Sudan Black Preparations* The most constant finding was a juxtanuclear clump of sudanophilic material. This appeared as a cluster of vacuoles (sudanophobic) in contact with solid sudanophilic spheres, or surrounded by sudanophilic rings or crescents. The majority of these clusters seemed to behave as a unit although a few were scattered throughout the cytoplasm. Some faintly grayish filaments and small spheres or granules were also present. In nearly every instance the pyknotic nuclei had a remnant of what appeared to be a nucleolus. It was a very rare occurrence to find sudanophilic material trapped between the nucleus and the cover slip, or beneath it.

3 *Phase Microscopy* Unstained dry smears of yolk-sac and heart blood mounted in 10 per cent formalin were examined with dark contrast phase microscopy lenses ( $15A-\frac{1}{4}\lambda$  and  $25B-\frac{1}{3}\lambda$ ). There was a remarkable degree of correlation between the morphology and distribution of dark structures as seen with phase microscopy and those seen in Sudan black preparations. The main difference was that the sudanophilic structures varied in color from faint gray to blue black, whereas the dark contrast structures appeared uniformly black. At no time did sudanophobic structures appear dark with phase microscopy. Pyknotic nuclei showed much more structure with phase microscopy than they did in May-Grunwald-Giemsa preparations. Nearly every nucleus contained one or more bodies which appeared to be the remnants of nucleoli. The large, non-nucleated primitive erythrocyte—not unlike some definitive erythrocytes<sup>2, 3</sup>—frequently contained quite a few organoids.

4 *Supravital Studies* Mitochondria in cells freed from yolk sacs of 15 day rat embryos did not stain as intensely as they did in cells from 11 day rat embryos,<sup>1</sup> and very often they appeared more finely divided. Although some filaments were faintly green, the color was uniform throughout their entire length. In neutral red-dahlia preparations, the neutral red was usually limited to a juxtanuclear cluster of vacuoles. Sometimes one or more of these vacuoles would also be found elsewhere in the cytoplasm. In successful preparations the dahlia would stain rings, crescents and spheres associated with the neutral red vacuoles. When pressure was applied to the cover slip, the cells moved about in a rolling fashion. In such cases the juxtanuclear clump of neutral red vacuoles behaved as a unit, maintaining their relative position within the cell and with respect to each other.

5 *Comment* It was reported previously that freshly dried films of primitive erythroblasts from 11 day rat embryos preserved cytoplasmic detail to an extraordinary degree.<sup>1</sup> Preliminary studies indicate that the same also obtains for blood from the 15 day rat embryo. Structures in dry smears which appear as sudanophobic vacuoles, or as light vacuoles with dark contrast phase microscopy have the ability, in supravital preparations, to segregate a basic dye like neutral red. Some of these vacuoles may be surrounded with a sudanophilic ring or crescent which occasionally stains with dahlia in supravital preparations.

This staining in the nucleated primitive erythroblast with neutral red differs from that originally studied in the erythrocyte by Israel and Pappenheim<sup>1</sup> and more recently by Ralph<sup>3</sup> in that these vacuoles were not newly formed but pre-formed

structures demonstrable in the unstained dry fixed smear mounted in 10 per cent formalin and the living condition with phase microscopy Dustin<sup>10</sup> has reported similar findings in erythrocytes of *Rana* and *Axolotl*, and he has identified these vacuolar structures with the Golgi element Since preliminary studies on 15 day rat primitive erythroblast show that preformed vacuoles are surrounded by argen-tophilic as well as sudanophilic material, it seems that the mammalian primitive erythroblast has a Golgi element similar to that described for erythrocytes of lower vertebrates <sup>10 11 12</sup> Such a Golgi element or apparatus is certainly not an artifact produced by over osmification as claimed by Ralph<sup>3</sup> for erythrocytes of the lower vertebrates

Newly formed neutral red vacuoles not associated with these juxtannuclear structures were present sometimes Dustin<sup>10</sup> has shown that these vacuoles appear when nucleoprotein is present Apparently when neutral red acts on the cytoplasm of cells containing basophilic substances in a diffuse state, the nucleoproteins become partly separated in a newly formed liquid phase and are segregated along with the dye in vacuoles This will be considered again in section B 2

### B *Smears of 15 Day Rat Embryonic Liver*

1 *Phase Microscopy* Until the present, all previous observations had been made on primitive erythroblasts In order to determine whether or not the same conditions obtained for definitive erythroblasts (the second or normoblastic generation of red cells), unstained dry smears of embryonic livers mounted in 10 per cent formalin (4 per cent formaldehyde) were examined A cursory examination of this material with a brightfield microscope revealed cells laden with numerous organoids, which were trapped between the nucleus and cover slip Figure 1a shows the best detail which could be brought out by reducing the condenser diaphragm aperture to N A 0.45 When this same cell was examined with bright and dark contrast phase microscopy, it was possible to bring out the finer details of these organoids This method showed that ring forms (like minute doughnuts) and filaments over the nucleus (i e, between the nucleus and cover slip) had the same morphology as organoids in the cytoplasm As shown in figure 1, b and c, some of the filaments extend from the cytoplasm beyond the nuclear membrane and continue over the nuclear area By careful focusing it was also possible to demonstrate that some ring forms had been trapped between the nucleus and cover slip over the nucleolar area It was also noteworthy that optical sections of nucleoli by means of phase microscopy appeared more like those seen in tissue sections after Zenker-formol fixation than they did those in dry fixed smears after methyl alcohol fixation

Similar observations were made on many cells in several preparations, one of which is illustrated in figure 1, d, e, and f This, like the one above, corresponds to those illustrated by Maximow<sup>13</sup> as large lymphocytes and cell 'A' (transition of a mesenchyme cell to pronormoblast) in the article by Kirschbaum and Downey <sup>14</sup> Such a cell is very interesting because all of the organoids in the thin layer of cytoplasm over the nucleus (i e between the nucleus and coverslip) are either ring or elliptical forms ranged in a column one behind the other in a depression between two nucleoli In the cytoplasm beyond the nuclear membrane, similar

forms are present but instead of being orientated on their edge or tilted, they are on their sides, giving the appearance of a circle or minute doughnut. These organoids are at a lower cytoplasmic level than those over the nucleus, consequently they are out of focus. In this cell, no filamentous forms are trapped between the nucleus and cover slip. In quite a few instances the filamentous forms beyond the nuclear membrane had a dilated terminal area which appeared to be forming the elliptical and ring-form organoids. Although this is beyond the scope of the present article, it would seem that the filaments are not increasing in length by the addition of

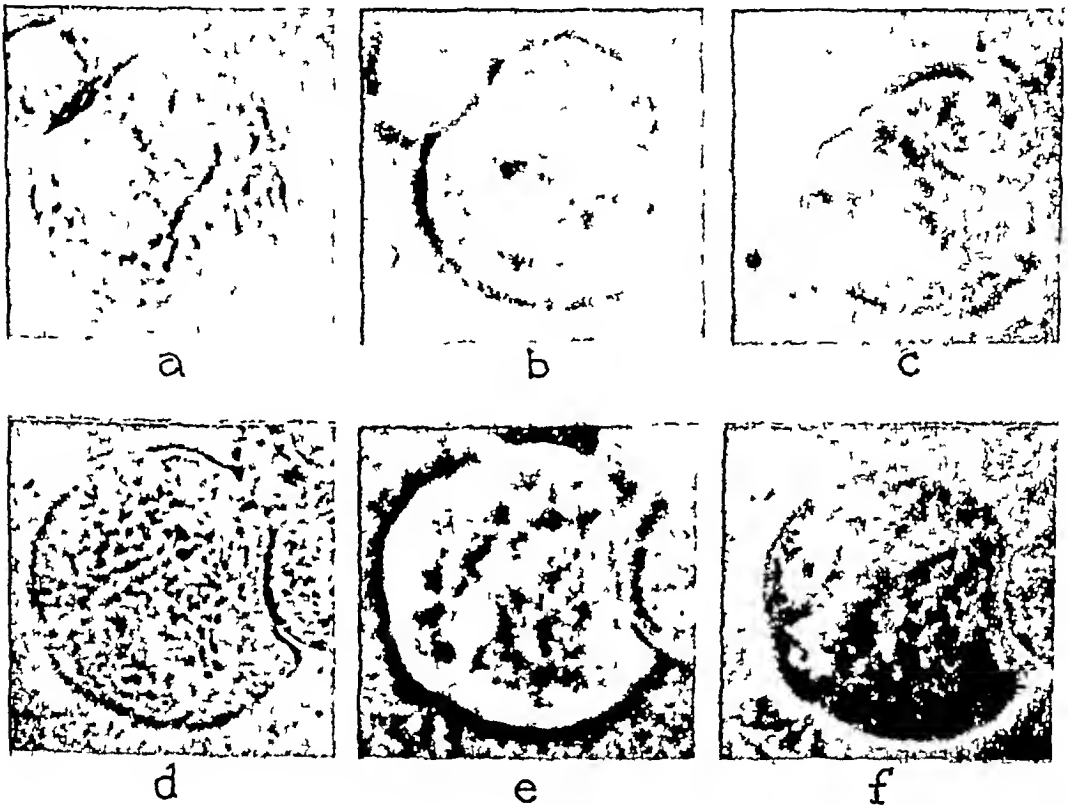


FIG. 1. Photomicrographs of unstained definitive erythroblasts (normoblasts) from 15 day embryonic liver mounted in 10 per cent formalin. Mitochondria are shown in layer of cytoplasm between nucleus and cover slip. Cells a and d, no phase, magnification 1835 diameters. Cells b and e, bright contrast phase, magnification 1820 diameters. Cells c and f, dark contrast phase, magnification 1820 diameters. All photomicrographs in figures 1 and 2 were furnished through the courtesy of the Research Division, American Optical Company, Scientific Instrument Division, Buffalo, N. Y.

material, but rather that they are becoming shorter by producing other forms. Granular and coccoid forms of organoids are also present.

The selection of 10 per cent formalin (4 per cent formaldehyde or Baker's formol calcium) as a fixing and mounting media was made on the basis that, after studying unstained cells by means of phase microscopy, they could be stained with Wright's stain or Sudan black. It is now known that the choice of this mounting media has the following additional advantages. First, phase microscopy works best when there are small optical path differences between the specimen and the surrounding

media.<sup>8</sup> Baker<sup>15</sup> has also pointed out that greater use should be made of aqueous media with a refractive index approximating that of water rather than ones in which the index is like that of glass. Another advantage in using this mounting media is that the cells in dry smears imbibe fluid and swell somewhat. This makes it possible to study more accurately optical sections through various cell layers above, through and beneath the nucleus.

Optical sections of numerous cells were made and studied with bright and dark

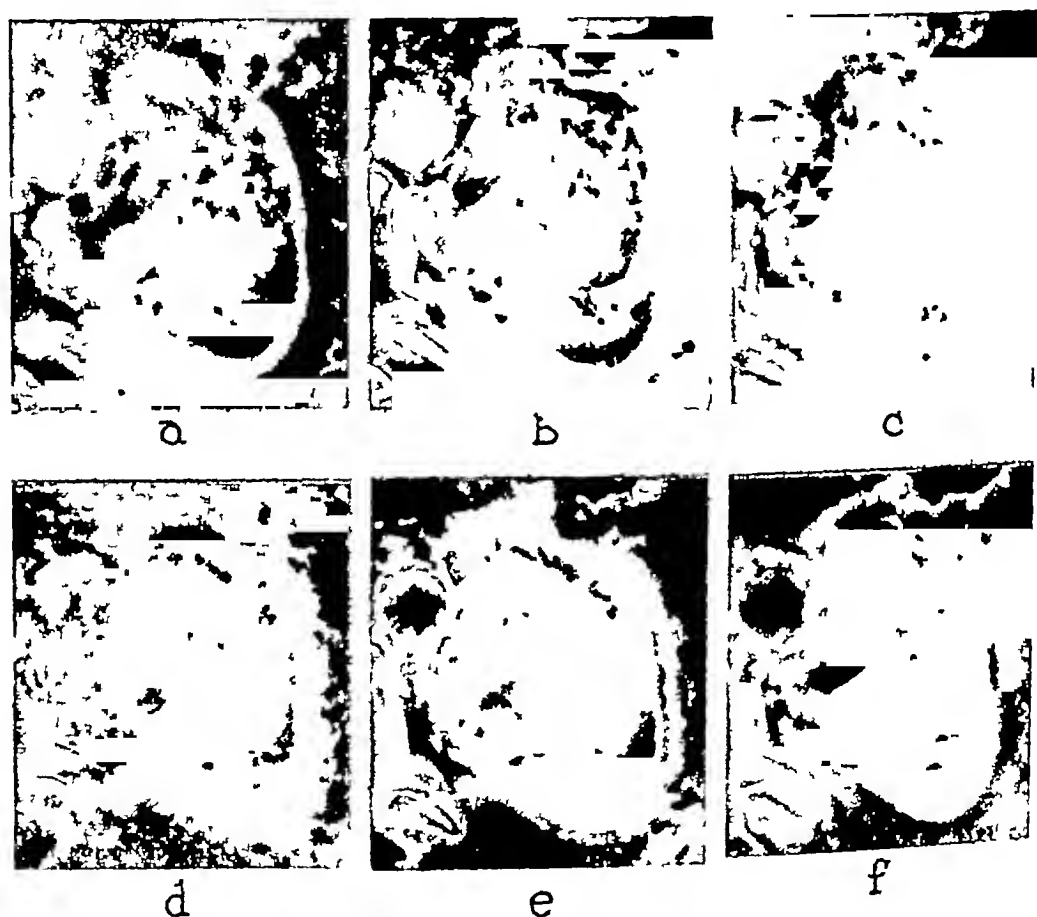


FIG. 2. Optical sectioning by phase microscopy of unstained definitive erythroblast (normoblasts) from 15 day embryonic rat liver mounted in 10 per cent formalin. Cells a, b and c show optical sections with bright contrast phase above, through and beneath nucleus respectively. Mitochondria appear bright. Cells d, e, and f show a similar series with dark contrast phase. Magnification 1820 diameters.

phase microscopy first through the thin layer of cytoplasm between the cover slip and the nucleus, then through the nucleus and finally through the thin layer on the other side of the nucleus. The cell in figure 2 is representative of this group. By using a fine adjustment control scale, these three levels had the following uncorrected vertical sizes, respectively 3, 2 and 4 microns. Optical sections through these levels showed that dry smears of lymphoid cells actually cause the nucleus to be sandwiched between two thin layers of cytoplasm which contain well preserved organoids. In figures 2b and 2e, the structures to the left and above the cell center

represent organoids out of focus which were actually in the layer of cytoplasm beneath the nucleus

A microscopist continually turns the fine adjustment control in order to bring as many structures into focus as possible and thereby gain perspective. Consequently, such a view is not well illustrated by a single photomicrograph. However, by making a camera lucida drawing it is possible to represent most of the structures found on either side of a given optical section. In figure 3, all of the drawings represent unstained cells as seen with dark contrast phase microscopy and correspond to some of the photomicrographs in figures 1 and 2. Drawings in figure 3, 1-c, 1-f

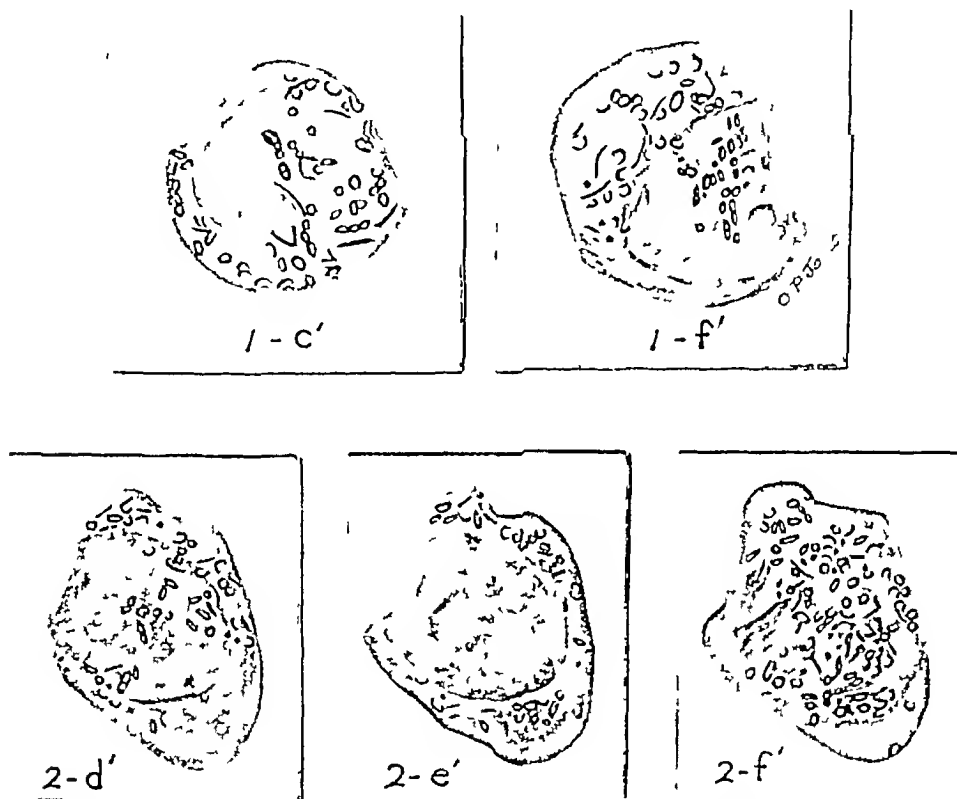


FIG 3 Camera lucida drawings of definite erythroblasts (normoblasts) shown by dark contrast phase microscopy in figures 1 and 2. Most of the organoids are mitochondria. Original magnification 2350 diameters, reduction about one-third.

and 2-d show all of the organoids in the thin layer of cytoplasm between the flattened nucleus and the cover slip. Dark contrast was so striking in these preparations that a casual observer might readily believe the preparations had been stained with either Sudan black or iron hematoxylin. Note that structures in the cytoplasm are in general similar to those situated over the nuclear area. In figure 3, drawings 2-e and 2-f depict what is seen in optical sections through and beneath the nucleus. It was surprising to find cell contour changed with various levels. This three dimensional variation is not appreciated in dry smears fixed in methyl alcohol. The important thing derived from these observations was that distribution of organoids within the two thin layers of cytoplasm is neither uniform nor equal.

However, there does seem to be a general rule governing the distribution of these organoids in lymphoid cells. The cells in figure 4, A and B-1, represent two adjacent

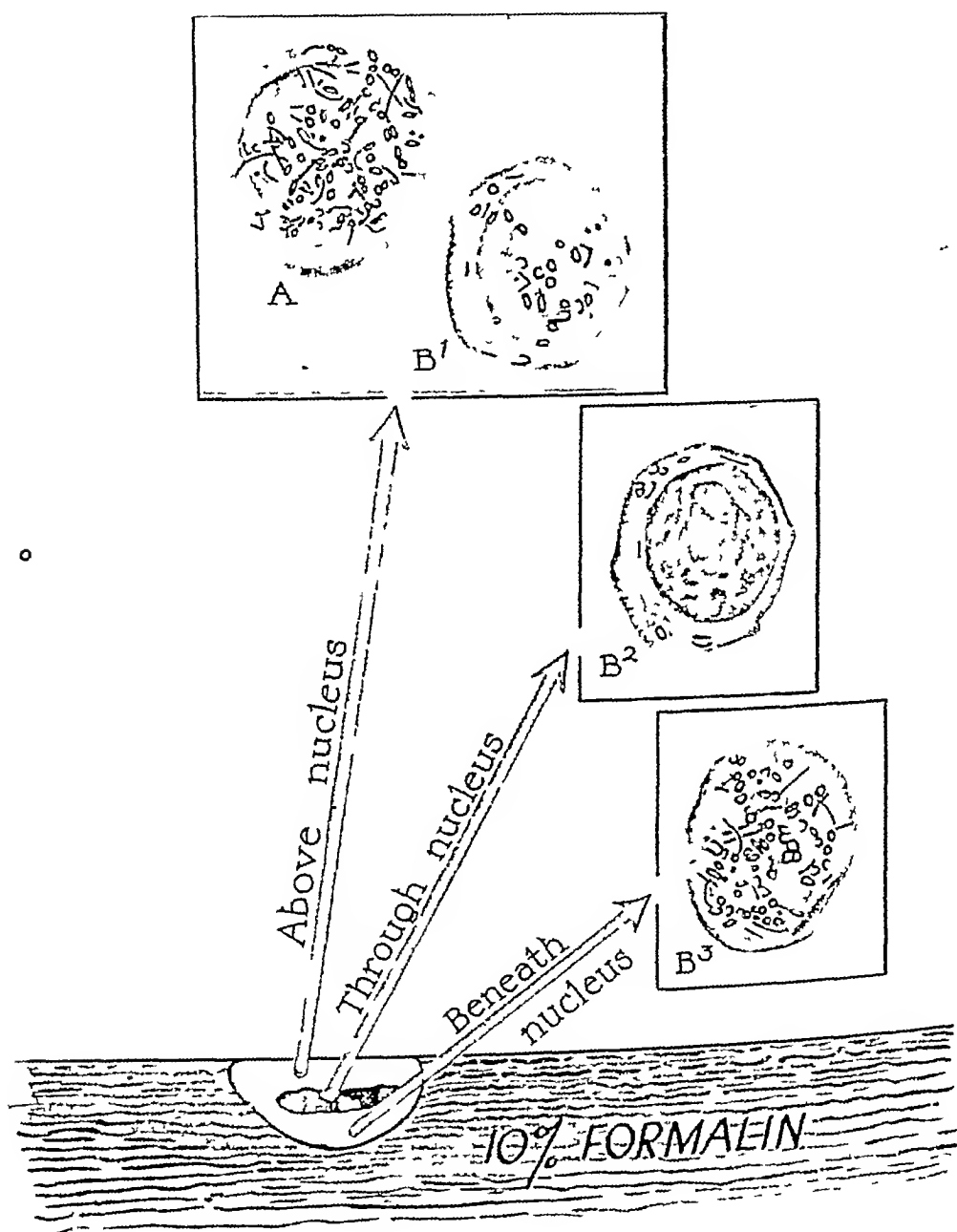


FIG 4 Camera lucida drawings of definitive erythroblasts (normoblasts) as seen with dark contrast phase microscopy in three optical sections. Cells A and B¹ show distribution of mitochondria in layer of cytoplasm between cover slip and nucleus. Cells B² and B³ show optical sections at different levels. Diagram illustrates cross section of erythroblast suspended from cover slip in 10 per cent formalin. Arrangement and legends were made by M. D. Diedrich, Director of Medical Illustration, University of Buffalo.

cells in which the distribution of organoids in the same optical sections differed inversely. In other words, the layer of cytoplasm above the nucleus in cell A is like the layer beneath the nucleus in cell B with respect to the amount of organoids.

An impression was gained, however, that perhaps more lymphoid cells had the majority of their organoids in the cytoplasm beneath the nucleus than above in coverslip preparations. Very likely the distribution of organoids depends upon such variables as degree of flattening, speed of the drying process and resultant thickness of the cytoplasmic layers. In an optical section through the nucleus, figure 3, B-2, one is again impressed by the appearance of the nucleolus. In figure 3, B-3, the optical section shows that layer contains about as many organoids as the higher level in cell A. The relative vertical sizes of these three levels were 2.5, 2.0 and 3.0 microns respectively. In other words, organoids are most numerous in the thicker layer of cytoplasm. It is very likely that the reason why the majority of cells have fewer organoids in the layer between the nucleus and the cover slip was due to the labile condition of the cytoplasm at the time the imprint or smear was made. Cytoplasm displaced from this position would tend to carry organoids around the edge of the flattening nucleus and distribute them in the thicker layer.

2. *Supravital Studies* Although phase microscopy makes it possible to see structures in unstained dry smears, the problem of identifying them and learning something of their nature remains. Hence, various dyes were used for supravital studies. The most successful of these was Janus green in dilutions of 1:20,000 and 1:40,000. Definitive erythroblasts (normoblasts) in these preparations were laden with green colored mitochondria corresponding in number, size, shape and distribution to most of the structures illustrated in figures 1-3. They were so numerous it seemed that little room remained for other organoids. This was particularly true of pronormoblasts and transitional cells between them and the mesenchyme. Ring-forms appeared as rods, ellipses or circles depending upon their orientation. The filaments were quite uniform. The number of mitochondria above and beneath nuclei was most impressive. So much so, that it is impossible to imagine how these cells could be smeared and dried without trapping many mitochondria in these locations. Practically all of these observations were made on the youngest cells and only slight consideration was given to the mitochondria in later stages of erythroblastic development. However, it was noted that the mitochondria tended to be more uniformly coccoid as maturation progressed. After one half hour, most mitochondria commenced to fade so that practically no green was visible by one hour after the preparation was made. However, in one sealed preparation kept at room temperature, the mitochondria remained colored for six hours.

Dahlia violet and neutral red brought out very few structures in preparations standing less than one hour. There was a slight indication that dahlia would tint some mitochondria. When the concentration of dahlia was increased to 1:10,000 a few dark solid spheres and crescents did take the stain rather intensely. These are probably portions of the Golgi element.<sup>4</sup> Neutral red vacuoles did not appear in the definitive erythroblasts as readily as they did in the primitive ones. After livers had been kept in the dye solution for one hour there was a sporadic and sluggish formation of neutral red vacuoles and granules. They were more numerous and larger in preparations kept for five to six hours. Dornesco and Steopoe<sup>11</sup> reported a similar difficulty in staining fish erythrocytes. One particular advantage in using fetal livers for studying erythropoiesis is that terminal developmental stages of



primitive erythroblasts are present in the circulating blood. Hence, in either dry smears or supravital preparations it is possible to study early definitive erythroblasts (pronormoblasts) as well as primitive ones in the later stages of their development. The latter develop neutral red vacuoles fairly rapidly in preformed structures before the former show any signs of reacting to the dye. When neutral red does appear in young definitive erythroblasts it does not seem to involve preformed structures. According to Dustin<sup>10</sup> this difference in the reaction of erythroblasts to neutral red is due not only to the presence of preformed vacuoles but to the condition of cytoplasmic nucleoproteins. In young erythroblasts the basophilia is stable but in those with considerable hemoglobin the basophilia is labile. For further consideration of this topic, see Dustin's discussion.<sup>10</sup>

3 *Sudan Black Preparations* In a previous study<sup>1</sup> it was shown that there was a good correlation between the distribution of mitochondria and the presence of lipid material. Furthermore, pictures obtained with Sudan black in primitive erythroblasts of the 11-day rat embryo corresponded very well to those of unstained cells seen with dark contrast phase microscopy. Hence, it was quite natural to expect that mitochondria in Sudan black preparations of erythroblasts in the 15 day embryonic liver might look like the cells in figures 3 and 4. Unfortunately, this was not the case. There was very little sudanophilic material in pronormoblasts of the second generation as compared with primitive erythroblasts from the 11 day yolk sac. Mitochondria appeared as very faint grey ring forms and filaments. In spite of this, it was possible to show that these organoids were distributed in the cytoplasm above and beneath the nucleus. If, as Lison and Baker claim,<sup>4, 16</sup> Sudan black demonstrates the distribution of total lipid, then mitochondria in these two cell types differ markedly in this respect. As to whether or not the exposure of these mitochondria to vapors of strong oxidizing reagents is necessary to saturate the unsaturated lipids and change them from a nonstainable to stainable form as Ralph<sup>3</sup> reported is not known at the present.

4 *Vitamin A* In a previous study<sup>1</sup> it was shown that the presence of vitamin A could be demonstrated in dry smears of embryonic blood and that it was localized in mitochondria. Similar studies were carried out on smears of the fetal liver. Vitamin A was present in the definitive erythroblasts (normoblasts) and in some of the polychromatic primitive erythroblasts. The reaction was fairly strong and could be localized in the ring forms as well as filaments.

5 *Permanent Mitochondrial Preparations* All of the evidence presented in the preceding sections indicates that most of the organoids in young definitive erythroblasts (pronormoblasts) are mitochondria. Additional evidence of a confirmatory nature was obtained in the aniline acid fuchsin preparations. Fuchsinophilic bodies—ring forms and filaments—were in the cytoplasm as well as over the nucleus. For the most part they were smaller than those in supravital preparations and unstained smears mounted in formalin. Very likely this was caused by the rigorous fixation and subsequent heat treatment.

6 *Separation of Mitochondria from Fresh Erythroblasts* Since most of the mitochondria in young definitive erythroblasts (pronormoblasts) are ring, elliptical or crescent shaped, it would be interesting to determine whether or not they maintain

their shape after being removed from erythroblasts. Three preliminary experiments, patterned after the method used by Hoerr<sup>17</sup> for extracting mitochondria from adult guinea pig liver, have been carried out. Six 15 day fetal livers were dissected free from surrounding tissues and ground in a small mortar at room temperature. This material was then suspended in 3 cc of 0.85 NaCl cooled to 0° C and centrifuged at 500 r p m for one-half hour. Both the supernatant and sediment were sampled and examined by dark contrast phase microscopy ( $0.15\lambda - \frac{1}{4}\lambda$ ). The supernatant contained many organoids and the sediment contained some intact erythroblasts and organoids. The supernatant was withdrawn and centrifuged at 2100 r p m for one minute. A sample showed ring forms were present in top layer. The supernatant was withdrawn and centrifuged again at 3000 r p m for fifteen minutes. The supernatant still contained a few ring forms but most of them were in the sediment. The small packed mass was broken up in 5 drops of 10 per cent formalin. Some of this was placed beneath a cover slip and sealed with vaseline for examination by dark contrast phase microscopy.

Since erythropoiesis is so conspicuous in the fetal liver and since ring form mitochondria are numerous in erythroblasts, the chances are that the isolated mitochondria came more from erythroblasts rather than hepatic epithelium. It seems to me the important thing to be gained from this observation is that mitochondria—ring forms in particular—behave as discrete structures and resist considerable mechanical force. Unfortunately, however, filaments did not survive this process of isolation.

7 *May-Grünwald-Giemsa Preparations* The observation that nuclei of various young definitive erythroblasts were sandwiched between two layers of cytoplasm containing many mitochondria quite naturally led to the question of what was their appearance in dry-fixed smears stained with standard hematologic dyes. Since it was known that light areas in basophilic cytoplasm represent negative images of underlying mitochondria,<sup>1</sup> it was felt that a somewhat similar picture might be obtained over the nuclear area. Before describing these recent observations, there are a few points which should be mentioned with respect to work previously done on human fetal livers.<sup>17</sup> In both previous and present studies, the methods of preparing and staining dry imprints of fetal liver were identical. In 1935, my attention was focused almost exclusively on the chromatin—its size, shape, distribution and staining affinity.<sup>18</sup> The same light areas which are about to be described and discussed were all considered as parachromatin—interchromatic nuclear spaces. My most extensive description was as follows. The parachromatin is faintly acidophilic producing a light pink tinge. However, in the case of promegaloblasts from pernicious anemia bone marrow the description was slightly more detailed.<sup>18, 19</sup> It was that, There is quite a large amount of parachromatin which is in the form of curved areas. The small masses of chromatin which are separated by curved areas of parachromatin are uniformly distributed throughout the nucleus.

An examination of the light areas in the basophilic cytoplasm showed that there was considerable variation in their size, shape and distribution. This is illustrated in figure 5a, which represents a transitional stage between the mesenchyme and

pronormoblast In the area between the nuclear membrane and the arrow there are negative images of mitochondria having the shape of filaments, short rods, spheres and ring forms No forms are present in this area for which counterparts cannot be discovered over the nuclear area This is also true for the reticular lymphocyte

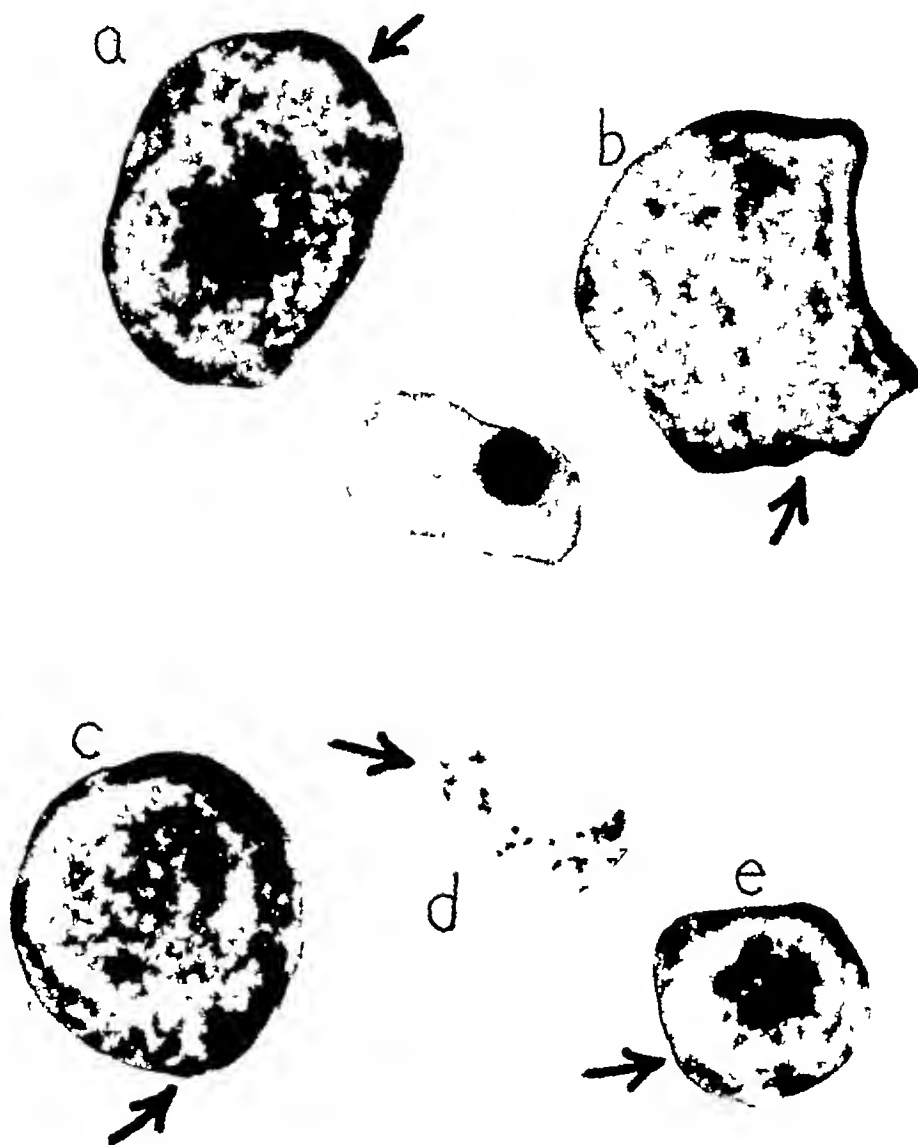


FIG 5 Photomicrographs of definitive erythroblasts (normoblasts) as seen in dry smears stained with May-Grünwald-Giemsa Magnification 1850 diameters Unlabeled cell is a polychromatophilic primitive erythroblast for comparison All others are young definitive erythroblasts (normoblasts) from 15 day embryonic rat liver Arrows point to light areas—negative images of mitochondria—discussed in text Photomicrographs were made by M D Diedrich

illustrated by Sundberg and Downey<sup>20</sup> in their figure 9 The difficulty, however, lies in the fact that contrast between the negative images and their surroundings in this location is not as great as it is in the basophilic cytoplasm that lies beyond the nuclear membrane This unquestionably is one of the reasons why hematolo-

gists have never or rarely doubted that all of the so-called parachromatic areas were entirely of nuclear origin. The other reason, of course, is that hematologists did not know freshly dried blood smears preserve cytoplasmic detail to an extraordinary degree.<sup>1</sup>

If some of the so-called parachromatic areas are of cytoplasmic origin, then it should be possible to find cells with negative images of mitochondria straddling the nuclear membrane. The cell in figure 5b is of interest because a filamentous light area extends from the cytoplasm beyond the flattened nucleus, across the nuclear membrane and continues over the nuclear area. In this cell, ring forms were also trapped between the nucleoli and cover slip. In figure 5c the arrow points to a cytoplasmic area in a pronormoblast which contains quite a few ring and elliptical forms. Similar forms, negative images, may be seen resting on the edge of the nuclear membrane while others are over the nuclear area. If these light areas are described with respect to the chromatin, it may indeed be said that there is sharp demarcation between them. This is a characteristic which for many years has been claimed for young erythroblasts and other myeloid cells. Figures 5d and 5e represent either late pronormoblasts or early basophilic normoblasts. In figure 5d, opposite the pointer, there is a filamentous light area which extends from the cytoplasm that lies beyond the nuclear membrane and continues over the nuclear area. In figure 5e, a similar condition exists but instead of one there are three such areas. Similar negative images, presumably of mitochondria, are shown in figure 10 of the article by Sundberg and Downey.<sup>20</sup>

These observations indicate that not all of the so-called parachromatic areas in lymphoid cells studied thus far are of nuclear origin. Many of these light areas—differing in size, shape and distribution—represent the negative images of mitochondria which have been impressed into the nucleus by drying and fixation. In other words, the nuclei of these cells have at least two entirely different substances which are light staining: one the oxychromatin and the other negative images of organoids.

In a previous section (B 1) it was shown that the distribution of organoids, mitochondria for the most part, varied in the layers of cytoplasm on either side of nuclei of cells in dry smears mounted in 10 per cent formalin. When dry smears are stained with one of the Romanowsky dyes, methyl alcohol fixation produces some shrinkage, and mitochondria above and below the nucleus are impressed into it. Hence, in a critical morphologic examination, it might make a difference whether preparations are on slides or cover slips. In the former, a free and slightly convex surface is examined first, whereas in the latter it is the flattened surface in contact with the glass which is examined first. This might influence the appearance of optical sections just beneath these levels. In order to study both sides of these erythroblasts, small square cover slip preparations were mounted in clarite on larger 24 × 60 mm cover slips. Negative images of mitochondria over the nuclear area were studied and sketched to show their distribution. In some cells, which were transitions between mesenchyme and pronormoblasts, it was possible to show that the nuclear area had four distinct regions depending upon the shape and distribution of the mitochondria as represented by negative images. This gave the chromatin a differ-

ent appearance in each region. In some, the filamentous and short rodlike mitochondria made the chromatin appear as if it was broken up by crevices (The drawing of a hematopoietic reticular cell, figure 7 in an article by Sundberg,<sup>21</sup> has a similar pattern.) In others, the ring and elliptical forms gave the chromatin a chain or droplet effect. When ring forms were in a horizontal position it was possible to see chromatin through their central area. If these "doughnuts" were in basophilic cytoplasm, then their centers would appear blue. Quite naturally, if it were not known that dry smears preserve organoids which are impressed into the nucleus and appear as negative images, then it would be justifiable to describe these several chromatin patterns within a single cell as being intrinsically nuclear. Sundberg and Downey<sup>20</sup> have described myeloblasts and reticular lymphocytes with three or more types of chromatin arrangement within a single cell.

By turning these preparations over, the other sides of cells were examined. Differences between the two sides were so slight that they could not be ascertained without knowing which side of the cell was being examined first. The reason for this is very likely that the shrinkage which accompanies methyl alcohol fixation tends to decrease distances between organoids in the two layers of cytoplasm on either side of the nucleus. Hence, optical sections through the nucleus would include nearly all organoids impressed into the nucleus. It was felt, however, that negative images of mitochondria impressed into the nucleus on the flattened surface adjacent to the cover slip were perhaps better defined.

8 *Comment* Although all of the foregoing observations were made on erythroblasts from rat fetal livers, enough human fetal liver material was examined to show that in general the same conditions obtained. Lymphoid cells from adult rat lymph nodes were studied in supravital and dry smear preparations by brightfield microscopy, and in supravital and unstained 10 per cent formalin preparations by phase microscopy.

## DISCUSSION

Prior to 1900 all lymphoid cells were grouped together, since most hematologists were unable to find any differences between those of the marrow and lymphocytes in normal circulating blood.<sup>22</sup> Naegeli<sup>23</sup> was the first hematologist to recognize and describe a characteristic chromatin arrangement in marrow cells. This was a uniform netlike structure present in small as well as large myeloblasts. And, as Downey<sup>22</sup> pointed out, it is the most permanent and reliable characteristic of the myeloblast which has survived until the present. Accurate descriptive terminology was more or less lacking in the field of morphologic hematology until Pappenheim<sup>24</sup> set a standard which is still accepted by many hematologists. He defined spongoplasm, paraplasm (hyaloplasm), parachromatin (karyoplastin), and chromatin with respect to their appearance in dry smears. The first three were grouped together and called plastin substances because they differed from chromatin by never taking up methyl green. Pappenheim made the keen observation, which almost seemed to be a law, that tinctorial reactions of the paraplasm and parachromatin paralleled each other. When this was applied to erythroblasts, he maintained that since staining of paraplasmatic hemoglobin paralleled that of the parachromatin,

it suggested an intimate genetic relationship because the former grew and changed tinctorially at the expense of the latter. He concluded that parachromatin or the interchromatic nuclear spaces were not produced merely by transillumination of plasmatic hemoglobin of the plasmatic cell circumference (i.e., cytoplasm above and beneath the nucleus in a dry smear), because they were also found in the torn out and displaced erythroblastic nuclei. Although Pappenheim may have been in error regarding the exact relationship between light areas in the cytoplasm and nucleus, he at least doubted momentarily that parachromatin was entirely of nuclear origin.

Pappenheim, Schridde, Naegeli, Ferrata, Downey and others, including the present author, have repeatedly emphasized that dry-fixed smears bring out the finer structural details of the nucleus which are important for the identification and interpretation of lymphoid cells which have not developed specific cytoplasmic characteristics. It has been generally accepted by all of these men that descriptions of nuclear detail, pattern, or structure have referred to intrinsic characteristics of flattened nuclei. To be sure, specific and azurophilic granules, and Auer bodies may have been either over or under a given nucleus, but these presented no problem because they were visible and readily recognized as such. Some hematologists must have pondered the fate of the cytoplasm in a more or less spherical cell which was subsequently flattened and dried. Likewise, some hematologists must have wondered what happened to the organoids when cells were smeared and dried. These questions quite naturally had to remain unanswered until the development of proper cytologic, cytochemical and optical techniques paved the way for their exploration.<sup>1</sup> Even before these techniques were utilized, Dr. E. R. Hayes of our department suggested that a possible solution might be to make smears on celloidin or some plastic substance and section them.

The present study is the natural outgrowth of a problem originally undertaken to determine the nature of the so-called hyaloplasm.<sup>1</sup> Investigation of various lymphoid cells with leptochromatic nuclei has shown that the thin layers of cytoplasm on either side of a flattened nucleus contain organoids in variable quantities. These were impressed into the intervening nuclear material during the drying and fixing processes in making a smear or imprint of hematopoietic tissue. When stained with Romanowsky dyes, the organoids—chiefly mitochondria—appeared as negative images over the nuclear area, just as they did in the basophilic cytoplasm. Unfortunately, it was impossible to separate readily all light areas of cytoplasmic origin within the nuclear area from those produced by intrinsic properties of the nucleus—oxychromatin in particular. All light staining material within the nuclear area, as seen in dry smears, has been described previously as either parachromatin<sup>18-24</sup> or karyoplasm.<sup>21-25</sup> Since the evidence derived by studying blood cells with several techniques indicates that cytoplasmic organoids may also contribute to the production of these light areas, it seems necessary to define the term nuclear structure.

Fundamentally, the morphologic basis for nuclear structure during interphase should include the nuclear membrane, chromatin reticulum, linin, nucleoli, and karyoplasm or nuclear juice. It should be an interrelation of the parts as dominated

by the general character of the whole. For practical purposes, the distribution of chromatin may be determined by using suitable basic dyes. However, in order to ascertain the true distribution of chromatin, the Feulgen reaction for desoxyribose nucleic acid must be used to exclude the possibility of other nucleic acids taking on basic dyes. Recent studies by Mirsky and Ris<sup>26</sup> indicate there may be a definite chemical difference between basichromatin and oxychromatin. The proposed definition of nuclear structure would apply to cells seen in histologic sections, optical sections of moist fixed smears, living cells and dry fixed cells mounted in aqueous media for phase microscopy.

This definition would not apply to lymphoid cells in the present study because the appearance of their nuclei was altered by the presence of organoids—chiefly mitochondria—contained in the thin layers of cytoplasm on either side of flattened nuclei as seen in dry smears. These structures appeared as negative images impressed into both sides of flattened nuclei. The extent of this alteration depends upon the type, amount and distribution of organoids present at that particular time. The picture thus presented is a pattern in the nuclear area produced by flattening the nucleus between overlaying and underlying organoids within thin layers of cytoplasm. This pattern is an arrangement of the parts that suggest a design. Therefore, unless one is absolutely certain that the nucleus has not been so influenced, it would be more appropriate to speak of the nuclear pattern of cells as seen in dry smears rather than the nuclear structure. Of course, the nuclei of all cells will not be so influenced by organoids because as they mature, as in the case of erythroblasts, there is a gradual diminution of cytoplasmic constituents. Hence, when an orthochromatic normoblast is examined the chances are that its nucleus was flattened between two layers of cytoplasm containing very few or no organoids to influence the parachromatic pattern. There is another aspect of this problem which has not been explored, namely, that slight differences in nuclear structure as seen in sectioned material may become accentuated when these more or less spherical bodies are flattened. Nuclear pattern must include intranuclear as well as extranuclear or cytoplasmic components. This tends to offset Maximow's criticism<sup>27</sup> that, "The minute differences of the nuclear structure, seen only in dry smears, might very well depend merely on the differences of external conditions, encountered by the cell in the various places and the various stages of development of the body."

Although it is necessary to have phase equipment to duplicate the present results, such equipment is not essential to check in general some of them. For example, by using either oblique lighting, or decreasing the condenser diaphragm, or a combination of these, or by dark field illumination, it is possible to determine that certain organoids are above the nucleus in mounted unstained dry smears. These methods, unfortunately, tell us nothing regarding finer details of these structures. Furthermore, if inquisitive hematologists had been really interested in determining the fate of mitochondria in dry smears, they could have arrived at the same conclusions through correlative studies of supravital material, Schridde and Freifeld preparations (methods outlined in Naegeli<sup>28</sup>) and dry smears. For example, in the recent study<sup>1</sup> on the relation of mitochondria to the so-called hyaloplasm the major conclusions were drawn from dry smear, supravital, aniline acid fuchsin,

Sudan black and antimony trichloride preparations before phase microscopy was used. The latter, however, clinched the argument. It may be shown eventually that the dry smear method preserves mitochondria better than any of the accepted technics. At least it may now be said that good dry smears preserve mitochondria in lymphoid cells with an unexpected degree of accuracy.

It should not be difficult for hemopathologists and clinical hematologists to think also in terms of cytoplasmic activity when interpreting various cellular responses, because undoubtedly a number of differences in nuclear pattern which they have studied have been due in some measure to organoids above and beneath flattened nuclei. In other words, part of their interpretations of altered cellular functional activity probably have been due to cytoplasmic conditions superimposed on nuclear ones. Since cytologists have shown there is a greater correlation between cellular function and the cytoplasm than between it and the nucleus, it means that by studying nuclear patterns in lymphoid cells we have a reflection of the nuclear as well as cytoplasmic alterations due to functional or metabolic activity.<sup>6</sup> We have been studying cytoplasmic alterations unknowingly for a long time (fifteen years in my case) and have inadvertently credited the functional activity of lymphoid blood cells almost entirely to nuclear metabolism with little or no credit to the basophilic cytoplasm and its negative images of underlying mitochondria. This interpretation in no way detracts from the role the nucleus has as a primary center of control in cellular metabolism and the importance of its organization.<sup>29</sup>

The results of the present study indicate that the dry smear method of studying immature blood cells produces nuclear patterns formed by the sum total of nuclear and cytoplasmic alterations. It seems to furnish a more sensitive indication of what a cell is doing than any other single known technic. For years, the neo-unitarians have, in many instances, more or less bridged the gap between the unitarians and the extreme polyphyletists (supravitalists). The reason is now more apparent than ever before. The unitarians or monophyletists relied chiefly on the use of tissue sections, hence they studied nuclear structure. (In this connection, it should be noted that Mirsky and Ris<sup>30</sup> have recently isolated chromosomes from interphase nuclei of fish erythrocytes and lymphocytes of calf thymus.) The supravitalists have relied primarily upon alterations of the cytoplasm and very little upon nuclear structure. There is some doubt as to whether or not they even studied all of the structures in certain cells.<sup>1, 11</sup> Certain supravital dyes like pinacyanol stain the nucleus as well as the mitochondria but nuclear patterns are not produced because mitochondria are not impressed into the nucleus. The neo-unitarians and dualists have been observing and describing differences due both to the nucleus and cytoplasm, especially in lymphoid cells. It is to their credit to have recognized that something was producing differences in nuclear pattern, even though it evaded detection until new technics clarified the contributing role of the cytoplasm. The present studies offer an explanation why Downey,<sup>22</sup> and later Sundberg and Downey,<sup>18</sup> concluded that the dry smear or imprint method revealed minute but significant differences which are lost through the use of other techniques. But it must be remembered that there exists an extreme variability of the morphological characteristics of any one cell type.<sup>20</sup> As a consequence we must pay more atten-



tion to nuclear pattern than we have in the past and we must give even more consideration to the range of variation of these patterns Pappenheim<sup>31</sup> also recognized the importance of the latter

The concept developed in this paper that nuclear pattern is a reflection of nuclear and cytoplasmic alterations should be applied to other cells in other conditions Klemperer<sup>32</sup> mentioned that "Every discovery of a structural detail in the cell arouses the question as to its functional significance" He also deplored the fact that cytologic investigations on mitochondria require a highly developed technic The present approach may be useful in studying certain aspects of cellular pathology It would be instructive to determine how much of the nuclear pattern of immature leukemic cells is due to cytoplasmic constituents In this connection, it should be pointed out that Lewis<sup>33</sup> suggested the derivation of malignant cells from normal ones might possibly be due to cytoplasmic alteration Later, Potter<sup>34</sup> studied—extracellularly—certain enzymes associated with cytoplasmic components and as result advanced his enzyme-virus theory of cancer Shortly afterwards, Woods and DuBuy reported that virus diseases, variegational diseases and cancer in plants may be related through changes in mitochondrial equilibria Although metastatic tumor cells in hematopoietic tissues might prove to be suitable material for studying the role of the cytoplasm in the production of nuclear pattern, there may be some difficulty in adapting these methods to exfoliated cells from cancerous tissues <sup>36</sup> Dr Raymond Kibler<sup>37</sup> from our laboratory has already extended many of these studies to myeloma cells with considerable success Sundberg<sup>21</sup> reported that "The uniformity of nuclear structure in both myeloblasts and lymphoblasts might well be the result of repeated mitoses without subsequent differentiation" Since mitochondria are abundant in dividing cells, future studies may reveal that these organoids are responsible for that uniform nuclear pattern This may also explain why hematologists have observed that the demarcation between the chromatin and parachromatin becomes more distinct in immature lymphocytes The role of the lymphocyte in acute inflammation<sup>38</sup> should be reinvestigated It is very likely that the increased hyaloplasm is not due to inhibition or cellular edema, but rather to an increase in the number of mitochondria as a result of accelerated functional activity It would also be interesting to reconstruct the changes in lymphocyte nuclei as they transform to macrophages and determine the role played by the cytoplasm <sup>38</sup> And finally, dry smears of tissue cultures may be suitable for similar analyses <sup>39</sup>

#### SUMMARY

- 1 Lymphoid cells from rat and human fetal livers and adult rat lymph nodes were studied by means of various cytologic, cytochemical and optical technics
- 2 Freshly dried smears preserve cytoplasmic detail with an extraordinary degree of accuracy
- 3 Mitochondria, both numerous and pleomorphic, are preserved in the cytoplasm which lies beyond the nuclear membrane as well as in the thin layers of cytoplasm above and beneath the nucleus
- 4 In dry-fixed preparations stained with Romanowsky dyes, mitochondria

appear as negative images (light areas) in basophilic cytoplasm. These negative images within the nuclear area contribute to the formation of what hematologists in the past have described as parachromatin.

5 The dry smear method of studying lymphoid blood cells produces nuclear patterns formed by the sum total of nuclear and cytoplasmic alterations which reflect functional activity.

6 The meaning of the terms nuclear structure and nuclear pattern have been contrasted.

#### ADDENDUM

Dr. Arnold Lazarow recently called my attention to the fact that other cytoplasmic constituents have been preserved in air dried smears of hepatic tissue. Paul Ehrlich (*Ztschr f klin Med* 6 43, 1883) anticipated by this method the results found later by the more elaborate freezing-drying technique. Ehrlich observed the homogeneous distribution of glycogen in liver cells.

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# THE BONE MARROW ON STERNAL ASPIRATION IN MULTIPLE MYELOMA\*

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## INTRODUCTION

THIS STUDY of the bone marrow on sternal aspiration in multiple myeloma is concerned primarily with three main points (1) the type of cell or cells involved in the production of the disease, (2) the origin of this cell or these cells, and (3) cytologic criteria for the degree of malignancy.

As will be noted more fully later on, there seems to be no point of agreement on the first two aspects of this subject and almost no information whatsoever on the third.

An attempt will be made herein to elucidate all three of these issues insofar as a study of the available literature and material at hand permits.

All satisfactory sternal marrow smears available through February, 1947, were reviewed for cell type and indications of origin. These numbered seventy-one altogether. Fifty-one cases were studied completely with differential counts, as noted subsequently, and with attention to all morphologic detail. These observations were used in an attempt to correlate cytologic findings with clinical findings. Forty-three cases had been seen long enough ago to be included in the follow-up study for indications of degree of malignancy. This last group includes all of those cases seen at the Mayo Clinic through December, 1945.

## METHOD

The technics of sternal aspiration used have been various modifications of the Arinkin method<sup>1</sup> (using a Klima and Rosegger needle<sup>2</sup>) and have been fully described previously.<sup>3-6</sup> The present method employed is that used by Schleicher.<sup>3-5-6</sup>

One to 2 cc. of the marrow material was withdrawn with a dry sterile syringe from the gladiolus at the level of the second interspace after inserting the tip of guarded needle and stylet 1 to 2 mm. beneath the inner table of the cortex. In addition to the marrow blood withdrawn, small elements (units) of bone marrow are apparently withdrawn intact. The whole is placed in a paraffin-lined heparinized glass tube. The use of these small units permits fixed tissue technics to be employed on the same bone marrow from which volumetric studies and smears are made.

Smears alone were available for review in the large majority of instances but in the few cases in which tissue was also available and of additional interest this fact is indicated. In addition to a general description of the sternal marrow smears that were made on each case after a period of study, differential counts of 200 marrow

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cells were made. Further "differential counts" were conducted on 100 "myeloma cells" in each case in an attempt to develop an objective method for determining the degree of anaplasia or immaturity.

A small, 200 cell differential count was considered to be adequate for this study, (1) because a larger 1,000 cell count run as a pilot showed less than a 3 per cent variation in the predominant cells present, (2) because the limits of normal are so wide<sup>4, 7</sup> that only gross changes are significant, and (3) because quantitative relationships in these marrows are of secondary importance. The number of nucleated erythrocytes per 100 myeloid cells was noted. The "myeloma" cells were classified as old if the nuclear chromatin was fairly compact and the nucleus small (usually eccentric), tending to resemble the ordinary plasma cells, whether or not a nucleolus was present, although this was noted.

The intermediate stage cell, the type most often seen in multiple myeloma, usually had one or more large nucleoli and a vesicular, finer stranded nucleus with or without chromatin "dots" (small isolated, denser areas of chromatin). There was, in this group, less tendency toward eccentricity of the nucleus than in the old cells. The young or immature forms were those with a large nucleus, with a very fine chromatin skein, with or without nucleoli such as seen in the stem cell or the reticulo-endothelial cell. Here the nucleus showed the least tendency toward eccentricity. This division proved to be a workable, though, as might be anticipated, not an infallible one.

#### HISTORICAL REVIEW

The significant clinical feature and gross morphologic aspects of the disease now widely known as "multiple myeloma" were developed largely during the latter half of the nineteenth century from the time of Macintyre,<sup>8</sup> Watson, Bence Jones<sup>9</sup> and Dalrymple<sup>10</sup> in 1848 to 1850 to that of Wright<sup>11</sup> in 1900. Macintyre described a case, appropriately enough of a man, 45 years old, who was seen in consultation with Dr. Watson, in whose urine Bence Jones noted some abnormal protein matter and on whom Dalrymple reported the microscopic findings in two affected ribs. Subsequently, Rustitzky,<sup>12</sup> Kahler<sup>13</sup> and Bozzolo<sup>14</sup> added coherence to the clinicopathologic picture of the disease and stimulated the reporting of more cases.

Until 1900, when Wright<sup>11</sup> published the studies he had made on a case of multiple myeloma that had come to his attention in February, 1898, no one had linked the offending marrow elements to the plasma cell. Shortly thereafter, however, Christian<sup>15</sup> reported on 6 cases, all with this characteristic finding, although he later stated<sup>16</sup> that he considered the myeloma cell to vary within the limits of plasma cell on one side and myelocyte on the other. Since that time, 75 to 80 per cent of reported cases have been of the plasmocytic type. It was during these formative years and the next two decades that most of our concepts of etiology, cell type and histogenesis were laid down. This was done in the main by pathologists utilizing fixed tissue sections. Subsequently, some of the other more bizarre manifestations of the disease were emphasized, such as hyperproteinemia,<sup>17-21</sup> rouleau formation,<sup>4, 22-26</sup> myeloma cells in the peripheral blood<sup>23, 24, 27-31</sup> and associated amyloidosis.<sup>19, 25, 35</sup> Two of the most complete reviews of the problem

to date include those of Geschickter and Copeland,<sup>76</sup> and Atkinson.<sup>19</sup> The relationship of the diffuse multiple myeloma to the solitary myeloma, intraosseous and extraosseous, and 'plasma cell leukemia' has been discussed by many authors, a few of whom are noted.<sup>27-31, 33-35</sup> In 1929, Arinkin<sup>1</sup> introduced his method for bone marrow aspiration and since then more attention<sup>1-39-43</sup> has been directed toward this technique as a method of diagnosis and means of studying the histopathologic characteristics of this disease.

## COMMENT ON THE LITERATURE

### A. THE TYPE OF MYELOMA

It has been widely held since the concept of multiple myeloma was first formulated that several, if not all, types of marrow cells were involved in the production of the specific lesion. There were thus erythroblastic,<sup>44-46</sup> lymphocytic,<sup>47-48</sup> megakaryoblastic,<sup>41-49</sup> myeloblastic,<sup>45-46, 51-52</sup> and myelocytic,<sup>39-44, 46, 48-50</sup> as well as plasmacytic,<sup>1</sup> lipoblastic<sup>51</sup> and osteoblastic types.<sup>53-54</sup> Of the current texts in pathology or clinical pathology this opinion finds support among Bell,<sup>55</sup> Wood,<sup>56</sup> Moore,<sup>57</sup> MacCallum,<sup>58</sup> Oertel,<sup>59</sup> Gradwohl,<sup>60</sup> and Kolmer and Boerner<sup>61</sup> and further acceptance by other authors.<sup>16-37, 62-77</sup> Boyd<sup>78</sup> seems to support those whose contention it is that all types are but a variation of one cell type when he remarks (having previously listed the usually accepted types) that "on the other hand it is equally possible, indeed probable, that the apparent variety is merely due to anaplastic changes in one fundamental cell type."

By the time Wright<sup>11</sup> made his first observations incriminating the plasma cell in 1900, the following histopathologic diagnoses had been made on the lesions of cases accepted as multiple myeloma: lymphosarcoma, myelogenous pseudoleukemia, lymphadenoma, hyperplasia of marrow, vascular endothelioma, sarcoma, small round cells and lymphoid cells. In 1904, Weber<sup>17</sup> reviewed the literature (38 acceptable cases) and reported one of his own. This case he classified as a "myelocytic" myeloma. He also recorded a second previously reported case, which he designated as "lymphocytic" although he mentioned the presence of more than the usual amount of cytoplasm and occasionally eccentrically placed nuclei.

In 1910, Williams and associates<sup>79-80</sup> reported a case of plasmacytic myeloma and also reviewed the slides and tissue of a case reported by Moffatt<sup>81</sup> in 1905 as lymphocytic. It was their expressed opinion that this, too, was plasmacytic. And even as early as that time they observed that "this suggests that further research will show that the differences in the type of cell are more apparent than real, and are the result of differences in fixation, staining and description, or perhaps in degree of anaplasia." Shennan,<sup>52</sup> in 1913, also, in a good discussion of the early literature, reported 3 cases, the second of which he regarded as myelocytic, the other two probably being plasmacytic. At the same time he presented a classification which included "true myelocytes, premyelocytes or myeloblasts, lymphocytes, plasma cells and megaloblasts (erythroblastoma)." However, it is interesting to see that though Shennan outlined a rather inclusive classification he did express some doubt that such a wide variety of types exists. "It is questionable

whether all the different varieties of myeloma described in the literature of the subject can be clearly differentiated from each other. Possibly the apparent differences are due to the variability of one cell type."

In 1916, Vance<sup>46</sup> reported a case which he placed somewhere between an erythroblastoma and a myelocytoma. He listed ten authors who had observed myeloblastomas, eight authors who had reported lymphocytomas, six who had noted myelocytomas, Ribbert's<sup>82</sup> erythroblastoma, and several authors who had described plasmacytomas.

Wallgren,<sup>83</sup> in 1920, in a widely quoted review of the problem, expressed the opinion that a lymphocytic type does not exist, that newer methods of staining have done away with it, and that all myelomas are made up of cells of the same fundamental type which may, however, show certain varying stages of development, differentiation or degeneration. In 1928, Geschickter and Copeland<sup>36</sup> summarized the findings on 412 cases of proved multiple myeloma from the literature and reported on their findings in 13 of their own cases. They had this to say about myeloblastic and myelocytic types:

As for the so-called myeloblastic or myelocytic type of myeloma, we have repeatedly observed that concerning the same section two authorities will differ between these two terms. Ewing, in cases 8 and 11 in our series, used the term myelocytic or myeloblastic, while Bloodgood used the term plasma cell type. In running through a series of 12 proved cases in rapid succession, one finds that these 2 cases do not stand out especially as atypical and fit in well with the so-called plasma cell type, although superficially, the nuclei do not appear to be as typically spoke-like in arrangement. Many authors, in describing such cells in cases of multiple myeloma are uncertain whether to class these cells in the plasma or myelocytic series, and some of these authors have thought that the apparent difference was due to fixing or staining methods.

In some cases the differentiation was made on the basis of the oxidase reaction, and in other cases on the Unna-Pappenheim reaction for plasma cells. While the differentiation can thus be made in some cases, often it cannot be made either by oxidase reaction or by Unna-Pappenheim stain.

Wintrobe,<sup>84</sup> while noting that the "literature refers to myelomas of various types: myeloblastic, lymphoblastic, lymphocytic, myelocytic, erythroblastic and even hemocytoblastic and megakaryoblastic," said, "Plasma cell myeloma is the most common designation. There is a good reason to doubt the identification of the cells in many of the reported cases. Even many of those who have referred to plasma cells have admitted that those were atypical. Recent studies which have been aided greatly by the possibility of obtaining the tumor cells by sternal puncture, indicate that the myeloma cell is a peculiar form differing from any other cell type."

*Cases reported in which tissue studies were responsible for diagnosis.* Wood, Quinlan and Merrill<sup>85</sup> reported a case of multiple myeloma in which the patient was a boy 19 years old. Tissue obtained for biopsy was submitted to four pathologists for their opinion because of the infrequency with which this disease occurs in the first two decades of life. Wood, Warren, and MacMahon<sup>85</sup> expressed the opinion that this was a pleomorphic or atypical plasma cell myeloma, while Geschickter<sup>3</sup> stated that it was of the 'primitive myelocytic series.' Donhauser and de Rouville<sup>86</sup> in reporting four cases of multiple myeloma noted that two were of the

plasma cell variety and that one was lymphocytic, while the other was myelocytic. This last was felt by Parker<sup>86</sup> to be Hodgkin's disease.

The foregoing 33 cases (table 1), reported since Wright made his observations on the plasma cell character of this tumor, are cases in which the histopathologic diagnosis is other than of the plasma cell type. It should be emphasized that in all of these cases the diagnosis was based on the examination of tissue sections. It will

TABLE 1. *Selected Cases of Multiple Myeloma from the Literature which were Reported as of other than the Plasma Cell Type*

Author	Type	Cases
MacCallum <sup>8</sup>	Myelocytic	1
Ribbert <sup>9</sup>	Erythroblastic	1
Weber <sup>1</sup>	Myelocytic	1
	Lymphocytic	1
Permin <sup>4</sup>	Promyelocytic	8
Moffitt <sup>11</sup>	Lymphocytic	1
Shennan	Myelocytic	1
Vance <sup>12</sup>	Myelocytoma	1
Gunn and Mahle <sup>13</sup>	Megakaryoblastic	1
Geschickter and Copeland <sup>14</sup>	Myelocytic	2
Wood and associates <sup>5</sup>	Myelocytic	1
	Lymphocytic	1
Donhauser and de Rouville <sup>85</sup>	Myelocytic	1
	Lymphocytic	1
Perillo <sup>59</sup>	Myeloblastic	1
Batts <sup>90</sup>	Myeloblastic	1
Symmers <sup>11</sup>	Myeloblastic	1
Wilson <sup>9</sup>	Lymphoid	1
Feller and Fowler <sup>17</sup>	Blast cell	1
Rosenblum and Kirshbaum <sup>93</sup>	Lymphocytic	1
Slavens <sup>61</sup>	Myeloblastic	1
Rypins <sup>94</sup>	Myeloblastic	1
Jacob and Kahn <sup>95</sup>	Myeloblastic	1
Jenkinson and Foley <sup>96</sup>	Without plasma cells	1
Cantarow <sup>7</sup>	Granulocytic	1
Moore <sup>98</sup>	Myeloid	1
Schwartz <sup>99</sup>	Giant cell myeloma	1
Marchal and Maller <sup>100</sup>	Hemohistioblast	1
Stewart and Weber <sup>23</sup>	Lymphocytoma	1
Smith and Silberberg <sup>62</sup>	Hemocytoblastic	1
Mewburn and Vango <sup>72</sup>	Lymphoid	1

also have been noted that great difficulty frequently attends the classification of these cells cytologically from tissue alone and that competent pathologists may differ widely in their opinions of the same tissue. And this takes no cognizance whatsoever of those cases in which a diagnosis of other than myeloma entirely was made erroneously, as has undoubtedly occurred in some instances.

*Cases reported in which diagnosis was based on sternal aspirations.* The technic of sternal aspiration is of relatively recent origin, yet, even so, enough cases of mul-



TABLE 2—*Cases of multiple myeloma diagnosed by sternal aspiration*

Author	Type	Cases
Scott <sup>7</sup>	Plasma	2
Reich <sup>101</sup>	Plasma	1
Pearson and associates <sup>102</sup>	Plasma	1
Rubinstein <sup>103 104</sup>	Plasma	2
Ferrata and Storti <sup>105</sup>	Plasma	1
Vogel and associates <sup>106</sup>	Plasma	4
Markoff <sup>107</sup>	Plasma	1
Gordon and Schneider <sup>108</sup>	Plasma	1
Hertzog and Schleicher <sup>100</sup>	Plasma	3
Beizer and associates <sup>4</sup>	Plasma	9
Berger and Goodman <sup>110</sup>	Plasma	1
Brugman and Reich <sup>111</sup>	Plasma	1
Wells and Goldish <sup>112</sup>	Plasma	1
Russell and Jacobson <sup>113</sup>	Plasma	1
Thannhauser and Brereton <sup>114</sup>	Plasma	1
Foord <sup>26</sup>	Plasma	1
Váradı <sup>43</sup>	Plasma	5
	Myeloblastic	2
Fleischhacker and Klima <sup>42</sup>	Plasma	5
	Stem cell	1
Rosenthal and Vogel <sup>39</sup>	Plasma	12
	Myelocytic	1
Weissenbach and Lievre <sup>115</sup>	Plasma	2
	Histiocytoid	3
Young and Osgood <sup>116</sup>	Plasma	1
Diggs and Sirridge <sup>117</sup>	Plasma	55
Magnabosco and Francescon <sup>118</sup>	Plasma	1
Du Bois <sup>119</sup>	Plasma	1
Mondor and associates <sup>120</sup>	Plasma	1
Dreyfuss <sup>121</sup>	Plasma	1
Suarez <sup>122</sup>	Plasma	1
Curtze <sup>123</sup>	Plasma	3
Blackman and associates <sup>124</sup>	Plasma	1
Bauer <sup>125</sup>	Plasma	1
	Plasma	1
Marchal and Mallet <sup>100 126 127</sup>	Plasma	1
	Plasma	1
Schleicher and Fahr <sup>128</sup>	Plasma	2*
Brass <sup>129</sup>	Plasma	1
	Plasma	4
Erf and Herbut <sup>40 41</sup>	Plasma	3
	Other	1
Churg and Gordon <sup>130</sup>	Plasma	1
Schindler <sup>131</sup>	Plasma	5
Bge <sup>132</sup>	Plasma	1
Moss and Ackerman <sup>133</sup>	Plasma	1
Meyer, Halpern and Ogden <sup>38</sup>	Plasma	1

\* (overlapping)

Plasma cell type 140

Other types 10

Total 150

multiple myeloma have been diagnosed by this method to make a brief survey of available reports of interest (table 2)

Varadi<sup>13</sup> reported on 7 cases, 5 of which were considered to be typical plasma cell forms, the other 2 being myeloblastic. The illustrations suggest that they might also be more anaplastic, younger varieties of the myeloma plasma cell.

Of the 6 cases in which Fleischliacker and Klima<sup>12</sup> obtained sternal aspiration material, five were classified as plasma cell types and one as [sic] 'Stammzellen myeloma?'. The question mark is theirs.

Rosenthal and Vogel<sup>14</sup> reported on 13 cases, of which all but one were of the plasma-cell type. The exception, case 13, was called myelocytic, although 3 per cent of plasma cells were present in the marrow on two sternal aspirations. Two plasma cells pictured had three nuclei apiece and were vacuolated. In this case, three questions may be raised: (1) was this really leukemia with moderately increased plasma cells or (2) was this really multiple myeloma with a severe leukemoid reaction and, if so, would still another sternal aspiration (two were done) have shown a different spread of cells with more 'myeloma plasma cells,' and (3) would a diagnosis of multiple myeloma be justified in the complete absence of the 'myeloma plasma cells'? In the author's opinion the mere fact of a cell's preponderance does not establish a cell type.

Weissenbach and Lièvre,<sup>115</sup> in the 5 cases which they described, found 3 to be 'histiocytoid' in type, and 2 plasma cell-like, again their description of the 'histiocytoid' cells, 'with eccentric nuclei, nucleoli, basophilic cytoplasm and frequently vacuolated,' prompts the speculation that these cells were merely the unrecognized, more anaplastic forms of the "plasma cell."

Erf and Herbut<sup>10-41</sup> reported 7 cases of multiple myeloma. 4 were of the plasma cell type, 1 was designated as lipoblastic, 1 megakaryocytoid and 1 myelocytic. The myelocytic patient had osteolytic bone lesions alone to suggest multiple myeloma, whereas her age, sex, hepatosplenomegaly, petechiae, marrow (10 per cent myeloblasts) and blood (6 to 15 per cent myelocytes) certainly point strongly to a diagnosis of leukemia. In this instance, then, a leukopenic leukemia is scarcely excluded. The sternal marrow in the case reported as megakaryocytoid disclosed many typical single or double nucleated plasma cells. It was because of the 'many transitions from these to giant cells with as many as 22 nuclei' that it was considered to be megakaryocytoid. The mere hypernucleation of the plasma cells here would seem dubious justification for such a conclusion and certainly the absence of the "many typical single or double nucleated plasma cells" would have made the diagnosis of multiple myeloma itself suspect. The third unusual case involved a man forty-one years old who had bone tumors and anemia and died after a brief illness. No Bence Jones proteinuria was noted and his serum proteins were low. The sternal aspiration examination suggested to the authors a likeness to marrow fat cells though reference was made twice to 'many cells that closely resemble plasma cells.' It is not clear on just what ground—the presence of bone tumors, lipoblasts in the marrow, the resemblance of marrow cells to plasma cells or a combination of these—the diagnosis of multiple myeloma was made in this case.

If on the first two grounds alone, the evidence would seem rather tenuous, if on the third, then the myeloma could scarcely be other than a plasma cell type

Reviewing carefully, then, the 10 cases noted in which other than a plasma cell type was reported, in the light of the present study as will be subsequently noted, it is seen that one is probably a case of leukemia (Erf and Herbut's<sup>40</sup> case 5), one is doubtfully multiple myeloma (Herbut's and Erf's<sup>41</sup> case 6), one (Rosenthal and Vogel's<sup>39</sup> case 13) plasma cell myeloma with a leukemoid reaction versus myelogenous leukemia or myelocytoma," and a fourth (Herbut and Erf's case<sup>41</sup> 7) probably a plasma cell type, though not so reported. The other 6 are all reported as immature types, 1 with excess plasma cells, 3 with a description that fits well the more anaplastic plasma cell and another with illustrations which, if accurate, depict 'plasmablasts' as frequently seen in this series of sternal marrow examinations

Haden and Rumsey,<sup>68</sup> in 1940, reported on 16 cases in which sternal aspirations had been done. Individual cases were not reported as such, and hence will not be considered further here

The difficulties inherent in the establishment of a cytologic diagnosis from tissue sections are evident and are emphasized by the experience of Geschickter and Copeland<sup>36</sup>, Wood, Quinlan and Merrill,<sup>86</sup> and Ulrich.<sup>37</sup> This point gains added weight by the observation of Erf and Herbut's<sup>40</sup> case 4 which was designated as myelocytic from sections, although the sternal marrow clearly demonstrated that these were plasma cells. Scott and associates<sup>134</sup> noted that "plasmoblasts" are not easily distinguished by routine staining methods from any early form of the myeloid or erythrocytic series and that this is probably the cause of many of the errors made by the early workers in interpreting them as myeloblasts and erythroblasts. They also pointed out the unreliability of "specific" plasma cell stains and the oxidase reaction in determining cell type in this disease (as have numerous authors) because of the variability of the cell to any of these tests

Churg and Gordon<sup>130</sup> in the same vein stated that "many cases [of multiple myeloma] have been reported in which the predominant cell was thought to resemble not the plasma cell but the myeloblast. This was true of many elements in our own material. If these cells had predominated, the case might have been called by some an instance of myeloblastic myeloma. However, all transitional forms between these and other cells resembling plasma cells were present. This statement is true in other reported instances. Apparently, many of the so-called myeloblastic cells are variants, perhaps younger forms of the typical cell of multiple myeloma. It seems logical, therefore, to group all the cells under one term, myeloma cell." <sup>135</sup>

Doan<sup>136</sup> said: "A diffuse hyperplasia of cells more or less characteristic of the so-called plasma type make up the new growth. The cells may show differing degrees of maturity. The plasmablast may be sharply differentiated from the myeloblast, lymphoblast and monoblast by the characteristic filamentous mitochondria (Doan and Lewenstein, 1936, unpublished data)." And Jones<sup>137</sup> noted that others have reported myelomas composed of myeloblasts, lymphoblasts, lymphocytes and erythroblasts. Apparently this discrepancy is due to the un-

certain identification of these cells in section material Fleischhacker and Klima point out that since the advocacy of sternal puncture the cells encountered in myelomas have been predominantly of the plasma cell type. Since these multiple myeloma cells may be immature they may present a picture similar to myeloblasts."

## B HISTOGENESIS

Many of the early conceptions of the immediate precursor of the myeloma cell depended on the accepted concept of multiple cell types and many authors have stated that a myeloma cell might arise from a lymphocyte,<sup>29 48 62 69 86 92</sup> lymphoblast,<sup>45 48 56</sup> myeloblast<sup>45 46 48 51 52 63 66 69</sup> or erythroblast.<sup>78 41 45 48 63 66 69</sup> In addition, it has been conceived that the osteoblast<sup>51</sup> or osteoclast<sup>53</sup> might be the progenitor of the myeloma cell. This hypothesis has no adherents today and fails entirely to consider extrasosseous plasma cell lesions which may clearly precede multiple osseous spread.

The conception of the relationship of myeloma to the erythroblast has developed on very frail evidence, has failed of corroboration with sternal marrow aspiration, and may be dismissed also without further discussion (even though Meyer and associates<sup>78</sup> noted a confusing similarity between plasma cells and nucleated erythrocytes in their reported case of 'plasma cell leukemia'). Since the origin of the plasma cell from the lymphocyte has long been authoritatively advocated, it is not necessary to believe that lymphocytic multiple myeloma exists in order to postulate a lymphocytic derivation for it. This has its adherents.<sup>29 138</sup>

Michels,<sup>139</sup> in a detailed review of the morphogenesis, function and development of the plasma cell in 1931, discussed the early formulation of morphologic concepts, pointed out that amitosis leading to the formation of multinucleated cells is a 'frequent phenomenon' and enunciated four main hypotheses of plasma cell origin (listing their proponents). These include (1) 'a histogenous origin from connective tissue cells, including tissue lymphocytes, fibroblasts, clasmatocytes, resting wandering cells, adventitial cells, hemohistioblasts etc.' (Piney, Downey, Naegeli, Maximow and others), (2) a hematogenic origin from emigrated lymphocytes', (3) 'mixed origin from emigrated lymphocytes (monocytes) or pre-existent tissue lymphocytes,' and (4) an origin from immature blood cells (myeloblasts, hemoblasts—erythroblasts, granuloblasts) through aberration or abortion." Michels also mentioned Maximow's tissue culture work (1922-1923) which showed that in explants of lymphoid tissue plasma cells develop from local lymphocytes in the course of two days. Jackson, Parker and Bethea<sup>29</sup> noted the similarity of multiple myeloma to lymphomas and said 'The type cell (plasma) belongs beyond question to the lymphoid series, and the clinical picture finds analogies throughout its course in the pathologic and symptomatic picture of the lymphomata.'

In the recent literature, increasingly greater mention is made of the origin of these cells ('myeloma' or 'plasma') from the reticulum, to the point now where the hypothesis has gained such widespread acceptance as the statement, 'since the disease originates in the reticulum cell'.<sup>110</sup> Osgood<sup>140</sup> stated that 'it is commonly thought that plasmocytes develop from lymphocytes, but I have never

found any evidence of this from my studies of multiple myeloma, plasmocytic leukemia or marrow or blood cultures. It seems certain that they are a distinct and separate line of cells." Both Scott and associates,<sup>134</sup> and Kracke and Garver,<sup>141</sup> who shared this conviction, cited Doan<sup>136</sup> as holding the belief that the myeloma-plasma cell arises from the reticular cells of the bone marrow and general connective tissue, Churg and Gordon<sup>130</sup> "stress the probable origin of the myeloma cells from the reticulum of the bone marrow" and cited Klemperer<sup>142</sup> and Rohr<sup>143</sup> (as did Jones<sup>137</sup>) as pointing out that the "plasma cell" of the myeloma is an abnormal hematic cell, the origin of which may be traced to the primitive reticulum cell of the bone marrow. Cappell<sup>144</sup> said, in reporting 2 cases of multiple myeloma "It might be considered that the abnormal myeloma cells are derived originally from the primitive reticulum cells. Some have differentiated through a myeloma cell of the common type or have even been further transformed into the plasma cell type."

Miller<sup>145</sup> injected tuberculinoprotein into the peritoneum of rabbits and observed that the precursor of the plasma cell was the reticular cell and its development could be traced through the blast stage to the typical mature Marschalko-type plasma cell, and finally into the degenerative phase with Russell-body formation. Lowenhaupt<sup>146</sup> examined 12 cases at necropsy and found splenomegaly in all but one. The splenic follicles were separated by wide expanses of plasma cells. Because these germinal centers (and those in the lymph nodes) remained intact even when widely separated by plasma cells, she felt that a hypothesis of lymphoid origin was untenable. She felt that she could detect single plasma cells and clusters of plasma cells hanging from the "reticular framework" into the vascular stream and felt that a "histiocytic origin" was suggested.

Recently, too, Parsons,<sup>147</sup> in a study of irradiated and tumor-bearing mice, arrived at much the same conclusion with regard to the plasma cells in these nodes. She observed that plasma cells develop locally in many inflammatory processes but that in these experimental mice it was systemic response, provoked by an agent acting on lymphoid tissue generally and primarily on the reticulum. Reticulosis was an early and constant feature and occurred coincidentally with the disappearance of lymphocytes. She wrote

Examination has given no evidence of the development of plasma cells from lymphocytes, which may indeed be entirely absent from the glands when plasma cells are abundant and active. Mitosis of lymphoid cells is rarely observed, even in the germinal centres where the reticulum cells appear to be in active proliferation and when karyokinesis of these and of plasma cells throughout the glands is marked. Proliferation of reticulum seems to start at the periphery and to spread inwards to the medulla, the existing plasma cells being in direct contact with these areas and having little relation to the diminishing lymphoid tissue.

In the early stages such glands show strands of branching cells forming a close network and staining more deeply with pyronin than normal reticulum. The nucleus is frequently eccentric. Later a perinuclear pallor develops in many cells, the nuclei of which are more darkly staining and have become more eccentric. Around the hilum the intersinusoidal cords contain few coalescing reticulum cells but only free mature and immature plasma cells.

Maximow and Bloom (1928) relate the plasma cell to the hemocytoblast, which they consider identical with the lymphoblast. No evidence has been found that this free mesenchymal cell is the direct

forerunner of the plasma cell, since the cells responsible for the plasmacytosis seen in the glands of the experimental mice appear to be the fixed reticulum cells of the stroma and identical with Maximow's fixed undifferentiated mesenchymal cells.

Hertzog and Schleicher<sup>109</sup> again stated "that the myeloma cells arise from the reticulum. This slide shows myeloma cells arising from vascular adventitial cells considered undifferentiated reticulum cells." Gordon and Schneider<sup>108</sup> noted that Kracke and Garver,<sup>141</sup> and Curtze<sup>123</sup> had suggested that plasma cells originate from primitive reticulum cells. They then said "The changes found in the hematopoietic organs in this case lend support to the view expressed by Kracke and Garver. At least strong presumptive evidence of this is supplied by the atypical reticulum cell hyperplasia found in the marrow, spleen and lymph nodes, and the transitional forms between reticulum cells and plasmablasts." Closely related to the concept of reticulum cell origin is that concept which holds that either the hemocytoblast<sup>9, 62, 145</sup> or the hemohistioblast<sup>13, 116</sup> is the parent cell.

#### C. DEGREE OF MALIGNANCY

In more than 230 references, only a handful of observers have commented on such aspects of the disease as they believed influenced the prognosis or mentioned factors which might be so implicated. In Morison's<sup>149</sup> fourth case, the disease was of brief duration and was "relatively anaplastic." Kolodny<sup>74</sup> noted that "among plasmacytomata one encounters a small and large cell variety, the latter are said to be of more rapid growth and somewhat worse prognosis." Caylor and Nickel<sup>150</sup> suggested that the "myelocytic" myeloma is more malignant than the plasma cell type. Gordon and Schneider's<sup>108</sup> case showed a very bizarre, anaplastic and pleomorphic marrow picture. The patient survived only about six weeks from the onset of symptoms. Mallory<sup>151</sup> said "I have never seen a bone marrow with so many immature plasma cell elements suggesting something very acute and very malignant." Moss and Ackerman<sup>133</sup> remarked about the myelomas with large numbers of circulating myeloma cells "Thus the readiness with which plasma cells tend to enter the blood stream should be a measure of malignancy. Such a statement seems logical, but it has not been proven." Hertzog and Schleicher<sup>109</sup> in presenting 3 cases of plasma cell myeloma at a pathologic conference, stated that "the solitary types are of a high degree of malignancy," and "one can estimate to some extent the clinical degree of malignancy by the amount of secretory activity of these cells."

Nothing definite can be concluded from reviewing these brief remarks but the suggestion is present that pleomorphism, anaplasia and cytologic immaturity are associated with a shorter clinical course.

#### REVIEW OF CASES

In the normal bone marrow, plasma cells average approximately 1 per cent<sup>4, 7</sup> of the leukocytic series. In diseases other than multiple myeloma, such as chronic inflammations, granulomas, measles, roseola infantum, carcinoma, aplastic anemia, infectious mononucleosis, Boeck's sarcoid, cirrhosis, lymphogranuloma

inguinale, monocytic leukemia, periarteritis nodosa and so forth, plasma cells may occur in excess numbers in the blood and bone marrow. Ordinarily this does not give rise to confusion with multiple myeloma, particularly if the diagnosis is clinically apparent. However, in an indeterminate situation, in which the bone marrow smear is expected to be of positive diagnostic value, some question may arise whether these excess and often abnormal plasma cells are not a part of a myelomatous process. This can not always be settled unequivocally on one examination, particularly negatively. However, in the great majority of cases little doubt remains.

The type of cell customarily found in smears of sternal marrow in multiple myeloma most resembles, of the normally occurring bone marrow elements, the plasma cell. The characteristics which distinguish the myeloma cell from the normal plasma cell have been described frequently in the past,<sup>4, 7, 39, 84, 148</sup> and there is little that is new to be added to these descriptions. Nevertheless, setting down here again the observations made in these cases may prove useful in re-emphasizing the specificity of this picture as a pathologic, as well as a clinical, entity.

Sternal marrow aspirations in 51 cases were studied, 1 case twice with a three year interval, and 20 more subsequently reviewed after this study had been completed. These latter 20 cases will not be included except as has otherwise been noted.

The first sternal aspiration on a patient who had multiple myeloma was performed at the Mayo Clinic in 1939. In that year 2 cases were noted, in 1940, 5 cases were observed, in 1941, 3 cases, in 1942, 4 cases, in 1943, 6 cases, in 1944, 8 cases, in 1945, 15 cases, in 1946, 19 cases, and in 1947, at the time of this report, 9 cases. Follow-up reports were sought and obtained only on those patients studied who had been examined at the clinic prior to January 1, 1946.

#### GENERAL CONSIDERATIONS

The size of the cells found varied approximately from 12 to 60 microns, considering the longest diameter of the cell, multinucleated cells not included. This variation occurred within cases as well as between cases. In some instances a marked uniformity of size and shape was the rule (fig. 1), in some cases moderate variation occurred and in still others, very bizarre appearing cells (fig. 2) were observed, varying greatly in size and shape, with, however, almost indistinguishable gradations between stages.

The cytoplasm was abundant as a rule, even in the very immature cells, usually equaling the size of the nucleus and often exceeding it fourfold to fivefold. It stained deeply basophilic, when the usual Romanowsky stains were used, often staining more deeply at the periphery to form a sort of 'rim' (fig. 3). In color it varied from a deep, slaty, cloudy blue to a rather bright and somewhat clearer blue. The cytoplasm was somewhat granular or pock-marked in appearance and not a clear homogeneous medium as is seen in the lymphocyte. Occasionally a perinuclear light, clear area or Hof was seen but this was inconstant. The character of the cytoplasm was one of the most persistently uniform and striking features of these

cells which held them together as a class, and impressed one with their similarity to the normal plasma cell.

The nucleus was often eccentric and, in some cases, almost constantly so. Here again the relationship to the plasma cell was emphasized. However, at this point, nuclear similarity usually ceased. Most nuclei had a finer chromatin pattern than the plasma cell, and this usually contrasted sharply with the parachromatin. A few had well-differentiated nuclei with heavy chromatin blocking (fig. 1). Among the rest were fine reticular patterns (fig. 4), diffuse stranded chromatin (fig. 3), or a granular chromatin background surrounding islands of condensed chromatin "dots" (fig. 5). As the cellular differentiation decreased, the tendency to eccentricity seemed to be proportionately reduced, though this still did occur in obviously immature or poorly differentiated cells.

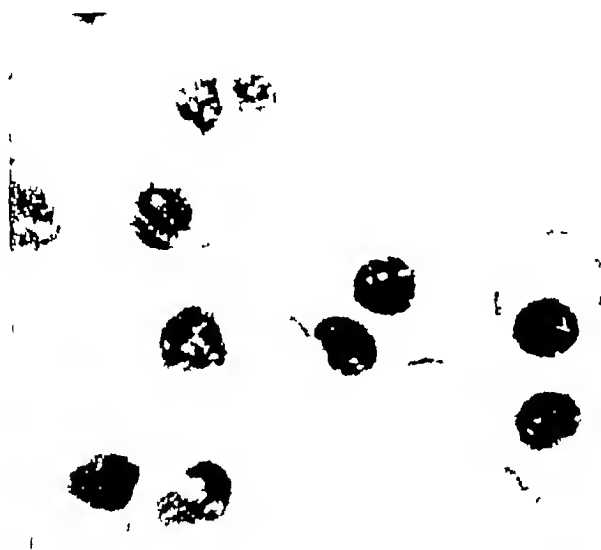


FIG. 1. Uniform, mature, differentiated picture in a chronic multiple myeloma. Patient living after eighty-eight months ( $\times 720$ ).

One, and occasionally more, large clear nucleoli were seen in the nuclei of most myeloma-plasma cells. These usually stained a pale sky-blue, lighter than the rest of the cell, although occasionally of a deeper blue. In all but two of the 51 cases studied there were more than 5 per cent of nucleolated myeloma-plasma (per 100 myeloma) cells. In six instances, nucleoli were noted in virtually all of the myeloma-plasma cells, and in about 57 per cent (29 of 51) of cases there were nucleoli in at least half of the myeloma cells present.

As might be expected, if amitosis were as common in myeloma-plasma cells as it is in normal plasma cells,<sup>139</sup> many multinucleated cells would be seen. This was so. At least one multinucleated cell was noted in each case studied, and the frequency rose to more than 5 per cent of all myeloma cells present. (The number of nuclei per cell was noted to exceed twelve, although two to three were more usual.) This



tendency to "giant cell" formation has led to various diagnostic difficulties, as these hypernucleated cells may give rise to the belief that they are megakaryocytes,

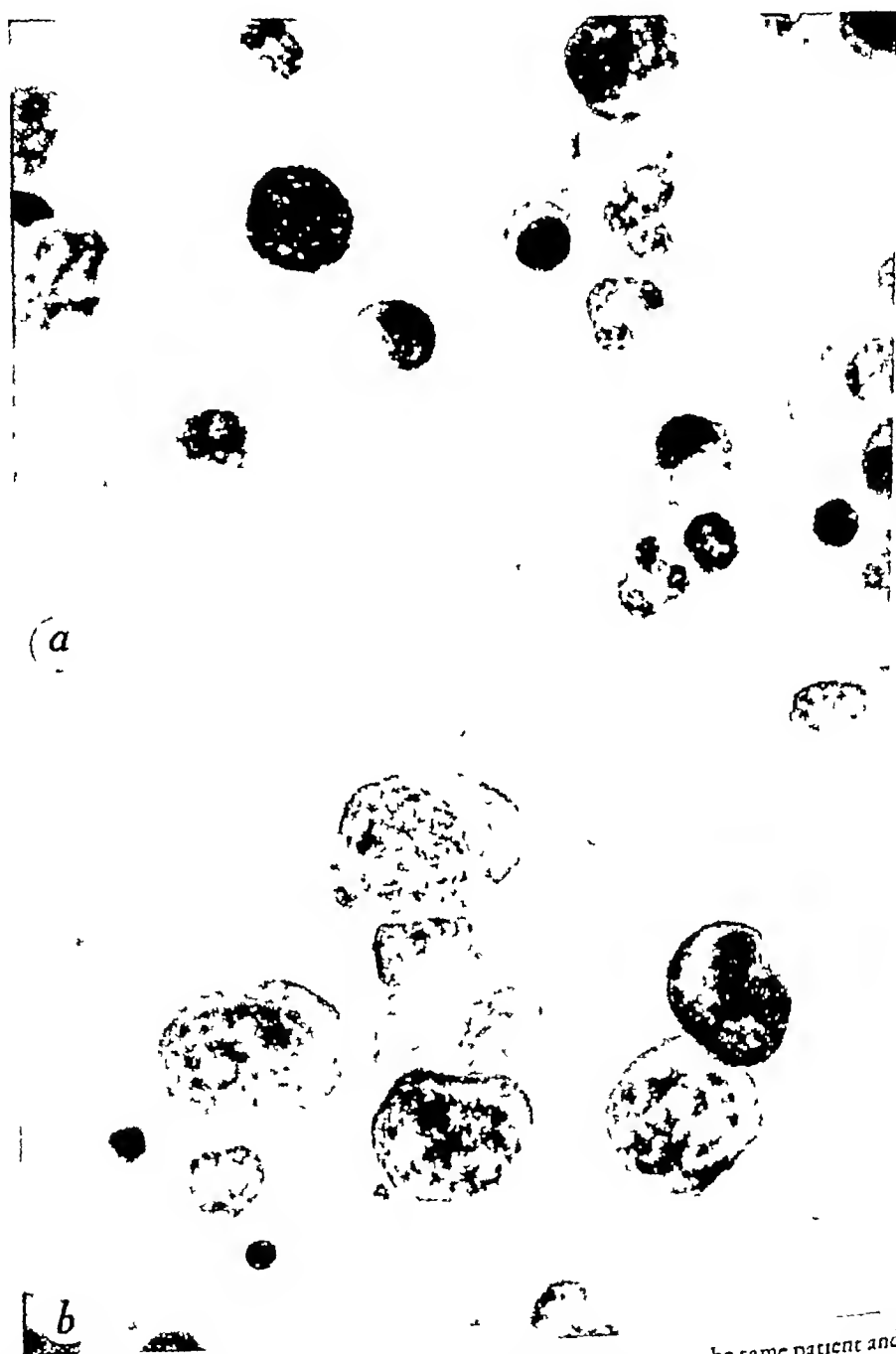


FIG. 2a and b These illustrations were both made from the same smear on the same patient and demonstrate vividly the degree of anaplasia and pleomorphism which may exist in the bone marrow in this disease ( $\times 725$ )

or (in tissue) tumor giant cells, or Sternberg-Reed cells. Mitoses were not common, but in those cases in which they were seen easily, the outlook was grave.

In all but 2 cases (rarely in 2 others) cytoplasmic extrusions were noted dis-

seminated freely through the marrow field. These extrusion bodies were identical in all respects with the cytoplasm of the myeloma cells. They stained alike, had

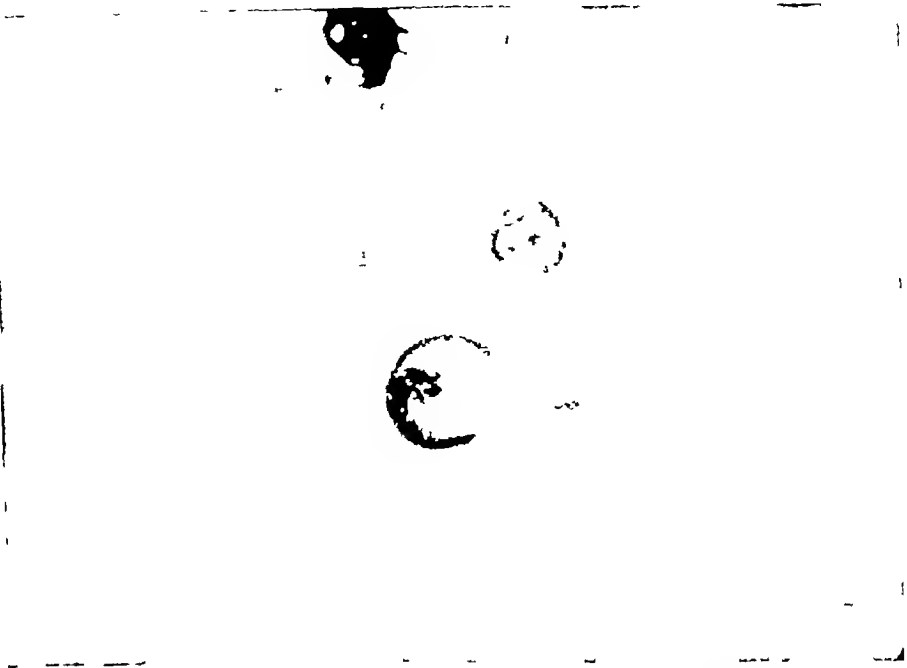


FIG. 3. Myeloma cells here constituted 2.5 per cent of leukocytic elements, lymphocytes 41 per cent. Note rim at periphery of myeloma cell ( $\times 900$ ).

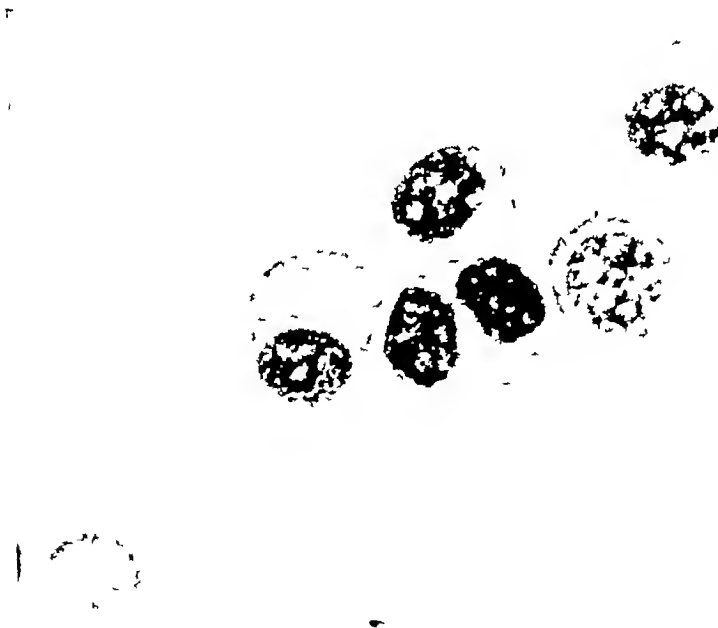


FIG. 4. MYELOMA CELLS WITH A RETICULAR PATTERN IN THE NUCLEUS ( $\times 720$ ).

the same internal granularity," contained vacuoles when the myeloma cells did and even contained Russell bodies in one case in which they were numerous. In

several cases they seemed to arise from buds or pseudopodia extending from the myeloma cell. These same extrusions have been seen in the peripheral blood in such

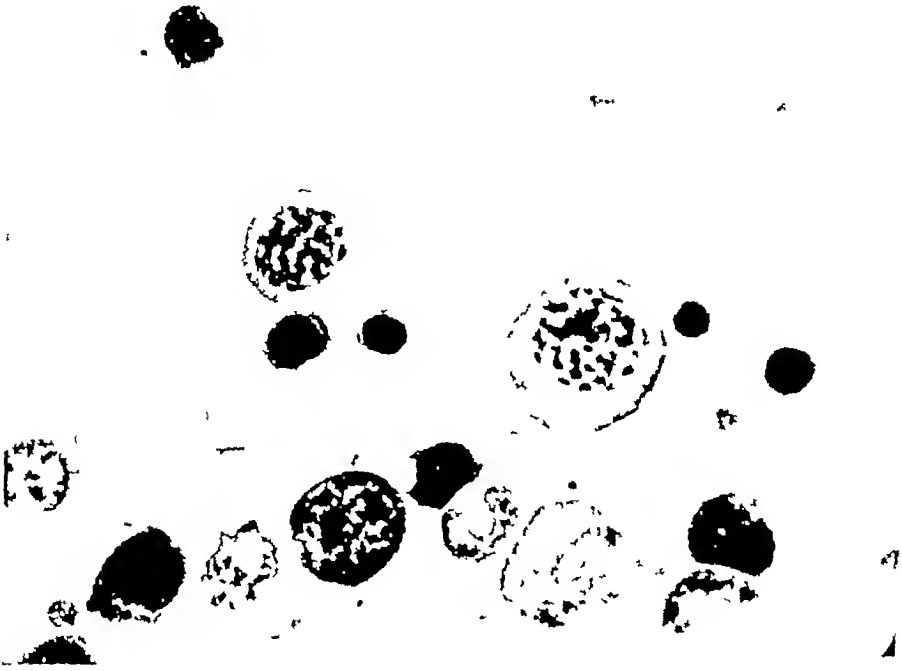


FIG 5 PROMINENT AREAS OF CHROMATIN CONDENSATION SINGLE NUCLEOLUS ( $\times 720$ )

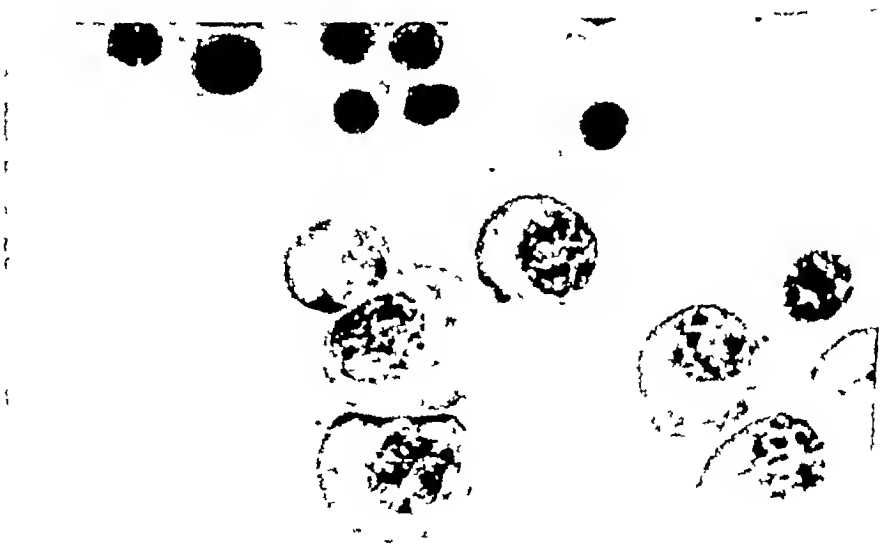


FIG 6 Russell bodies may be seen in the upper center cell. Smaller ones were present in the other cells but are not apparent here ( $\times 850$ )

cases. That this has special diagnostic value, however, is doubtful as the same phenomenon may be seen in other conditions, particularly the lymphocytic and mono-

cytic leukemias In this connection, it is of some interest to note that Sabin is quoted by Lowenhaupt<sup>146</sup> as showing that release of globulin by clasmotocytes is associated with the shedding of cell cytoplasm into the blood stream However, despite the attractive possibility that it might, no relationship could be noted between the rate of occurrence of these cytoplasmic bodies and the presence of Bence Jones proteins in the urine, or the degree of elevation of the serum proteins

Among the common degenerative changes, so-called, that occur in plasma cells, vacuolization was noted often in the myeloma-plasma cells In some instances, cells were literally riddled with vacuoles, and in at least two instances the nuclei were involved as well An effort to stain such nuclear vacuoles with amyloid stains met with negative results in a recent case in which this was attempted Appreciable vacuolization was noticed in more than half of the cases (29 of 51) Russell bodies (fig 6) were not as numerous, however, even in those cases in which they were seen, although there were two instances in which this was not so They were observed in 11 cases altogether, a trifle more than a fifth It may be mentioned in passing that as recently as 1937,<sup>148</sup> it was stated that Russell bodies had not been demonstrated in the myeloma cell

As might be expected, rouleaux formation in the sternal marrow aspiration was noted with approximately the same frequency as it was in the peripheral blood, 78 per cent

An attempt was made to correlate the cytologic findings with the presence of Bence Jones proteins in the urine, according to Hertzog and Schleicher's method,<sup>109</sup> or any other that might suggest itself No consistent cytologic property was noted, the occurrence of Russell bodies was without significance, as was the presence of vacuoles, or cytoplasmic extrusions The state of cellular differentiation likewise was immaterial

Schleicher<sup>109</sup> suggested that during the "phase of growth" in which the cells are uniform in size and the cytoplasm stains deep blue no Bence Jones protein will appear in the urine On this basis he correctly predicted that Bence Jones proteins would be absent from the urine in a case described "When the cells secrete globulins they become larger and vary in size The cytoplasm appears to be vacuolated and stains irregularly" Disappointingly, in several of our most monotonously uniform cases Bence Jones proteinuria occurred as it did in the case with the clearest, bluest cytoplasm Conversely in some of the cases with large, pleomorphic cells with deep, irregularly staining and vacuolated cytoplasm, Bence Jones proteinuria was not present Hence it has been impossible at present to duplicate Hertzog and Schleicher's results

#### CELL TYPE

Myeloma-plasma cells were seen in every case, now totaling 71, without fail and consequently a diagnosis of plasma cell myeloma was made in each instance There were no exceptions However, it should be noted carefully that the myeloma-plasma cell was not the predominant marrow cell in every case In fact, it was not the predominant cell in more than a quarter, or 13, of the cases Lymphocytes were present in equal or greater numbers in 11 of these 13 cases Figure 4 shows the char-

acteristic myeloma-plasma cell which was present in a smear in which 41.5 per cent of the cells were lymphocytes, while only 2.5 per cent were myeloma cells.

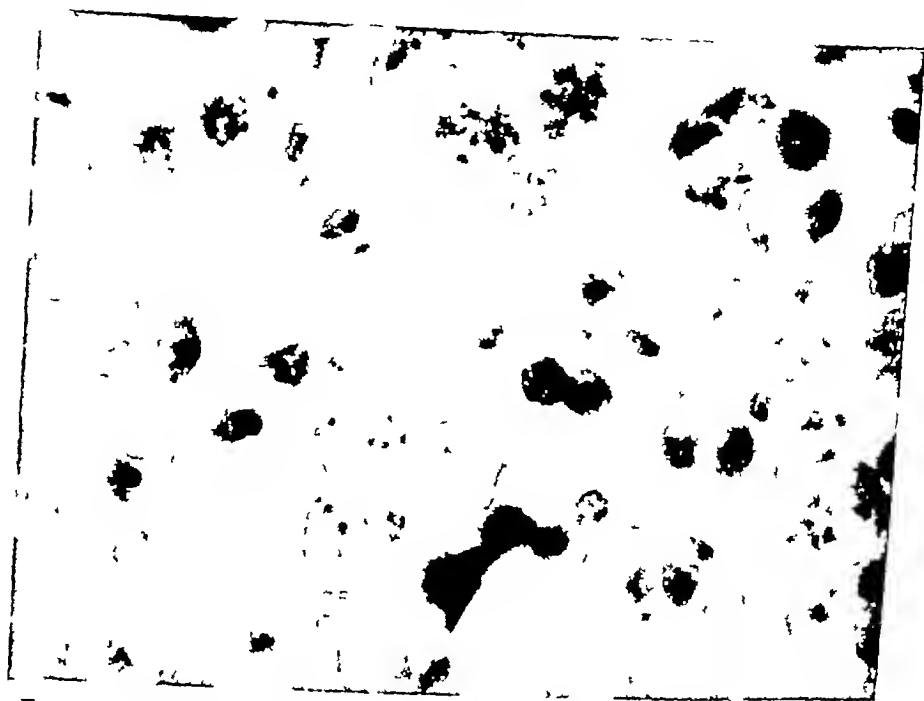


FIG 7 FIXED TISSUE SECTION FROM BONE MARROW SAME CASE AS FIG 2



Fig 8 Typical myeloma-plasma cells from a case interpreted as Hodgkin's disease on the basis of examination of fixed tissue ( $\times 850$ )

Of these, 39 of the 100 myeloma cells enumerated had nucleoli. This patient was a man, aged 50, who had suffered from low back pain associated with progressive

weakness and paralysis of his legs for a year prior to his admission to the clinic. Osteolytic lesions were noted in the roentgenograms of his thorax, thoracic and



FIG 9 BINUCLEATE MYELOMA CELL IN STERNAL MARROW ( $\times 1,000$ )

0



FIG 10 Typical myeloma cell from marrow, same case as figures 9 and 11 through 16. Prominent, single nucleolus ( $\times 1,100$ )

lumbar segments of the spinal column, pelvis and femora with compression of the eleventh and twelfth thoracic vertebrae. No test of his urine for Bence Jones pro-



FIG 11 More mature myeloma cell shows definite plasma cell characteristics now. Shrivelled nucleolus still can be made out at the 5 o'clock position ( $\times 1,120$ )



FIG 12 Cell very suggestive of reticulo-endothelial cell. The nuclear pattern is fine and skeinlike, there is no blocking of chromatin nor any evident tendency to condensation. The cytoplasm is pale. The outline of a large nucleolus may be seen. This and the following four cells were noted in the peripheral blood of the patient whose sternal marrow was illustrated in the preceding three figures ( $\times 1,000$ )

teins was made. The concentration of protein was 10.2 Gm per 100 cc of serum and albumin-globulin ratio was 1:3.9. The patient died at home four months later.

In 4 of the 11 cases mentioned, the lymphocytes exceeded the myeloma-plasma

cells by less than 5 per cent, which actually means that they occurred with approximately equal frequency in these cases. In the other 2 of the 13 cases, nucleated erythrocytes were present in numbers approximately three times as great as that of any other cell. In these 2 cases, characteristic myeloma-plasma cells were present to the extent of 16.5 per cent and 42.5 per cent of the leukocyte line.

The implications of this in the examination of fixed tissue should be apparent, namely, the possibility of designating the case as one of lymphocytic, erythroblastic, or myelocytic myeloma (here the oxidase reaction would be positive) on the basis of the predominant cell type.

Another of the hazards of tissue diagnosis is exemplified by the following case. A man sixty-five years old presented himself at the clinic with a three week history of back pain which he first noticed after climbing a fence. Rapid progression and deterioration ensued until the patient was disabled by his pain at the time of admission, just three weeks after its onset. He had been "completely well" prior to that time. An intensely tender tumor was noted medially in his right clavicle and roentgenographic examinations of his skull, thorax, and spinal column revealed multiple discrete osteolytic lesions. Bence Jones proteins were noted in his urine. His serum proteins were normal and the concentration of urea was 110 mg. per 100 cc. of blood. He died at home within the following week.

Examination of sternal marrow smears from this patient showed an extreme degree of pleomorphism (fig. 2). Eighty-five per cent of the myeloma cells present were immature or poorly differentiated and virtually all contained nucleoli. Although the more differentiated cells (15 per cent) in the smear (fig. 2a) clearly established the identity of these cells, sections of tissue secured at the time of sternal aspiration would have been called "myeloblastic" (fig. 7), if, indeed, a diagnosis of multiple myeloma had been sustained at all.

More recently a 48 year old man appeared at the clinic with a four month history of thoracic and back pain, and severe anemia. His liver was enlarged and he had axillary adenopathy. On roentgenography his skull, ribs and spinal column were observed to be involved with multiple punched-out osteolytic lesions. The concentration of hemoglobin was 4.35 Gm. per 100 cc. of blood, erythrocytes numbered 1,390,000 and leukocytes 3,800 per cubic millimeter. Excessive rouleaux formation and promyelocytes were seen in smears of his peripheral blood.

Albuminuria was graded 2 to 3 (on the basis of 1 to 4, in which 1 designates the mildest and 4 the most severe condition), but the urine was negative for Bence Jones protein on two occasions. The erythrocyte sedimentation rate was 172 mm. in an hour (modified Westergren) and the total proteins were 9.2 Gm. per 100 cc. of serum. He had an albumin-globulin ratio of 1:1.51, and a concentration of urea of 82 mg. per 100 cc. of blood.

Sternal aspiration revealed a typical plasma cell myeloma of a poorly differentiated type with 30.5 per cent of myeloma-plasma cells with 2.5 per cent multinucleated (fig. 8). However, at necropsy, the examination of the fixed tissues presented a picture which was interpreted by the pathologist as being that of Hodgkin's disease, including a heavy reticulum and Dorothy Reed or Sternberg-Reed cells (that is, multinucleated cells with two or three nuclei).



It may be seen, then, that though the degree of plasma-cellular differentiation may vary strikingly between cases and even within cases, a strong bond of cytologic similarity exists between all cases, and the transition from cell to cell can be observed to proceed through scarcely perceptible gradations. Three important pitfalls to proper diagnosis were recognized (1) immaturity, (2) multinuclear plasmagiant cells, and (3) lack of preponderance of myeloma cells in some cases.

#### HISTOGENESIS

One looks to the more differentiated cells in a tumor to indicate its destination, and to the least differentiated cells to reveal its origin. In the immature myeloma cell, a granular, finely reticular (leptochromatic), skeinlike chromatin is frequently



FIG. 13. Here is a cell almost identical with that in figure 12 with two important exceptions (1) the nuclear pattern is becoming heavier, and (2) a tendency toward condensation of the chromatin is suggested. A large nucleolus is readily seen ( $\times 1,000$ ).

encountered. In certain cases (fig. 4), seen even in the late phases, it is strikingly apparent. This in itself would seem to be strong support for the hypothesis that the myeloma cell is derived from the reticulum, as has been so often stated by others and noted earlier in this paper.

Further strengthening this conclusion are the findings in the following case which I believe is, in many ways, unique. When first examined in the spring of 1940, the patient, a woman 60 years old, had moderate hepatosplenomegaly, osteolytic lesions in her skull and extremities (the only regions of which roentgenograms were taken), repeatedly positive reactions for Bence Jones proteinuria, albuminuria, grade 4, moderately severe anemia and the peripheral blood picture of

leukemia. A sternal aspiration at that time showed 11.5 per cent of characteristic myeloma-plasma cells (figs 9, 10 and 11), and a diagnosis of multiple myeloma was made. A review of the peripheral blood smear taken at that time shows characteristic myeloma cells present as well as myeloid immaturity (leukemoid reaction). Subsequently, however, the patient's liver was found to "fill her whole abdomen" and the peripheral blood contained many cells almost indistinguishable from (if, in fact, they were not) reticulo-endothelial cells. These final developments were so marked as to cause the clinician to change the final diagnosis to reticulo-endotheliosis.

Repeated careful reviews of all the slides made on this case from the marrow and peripheral blood emphasize that myeloma-plasma cells were present from the start.

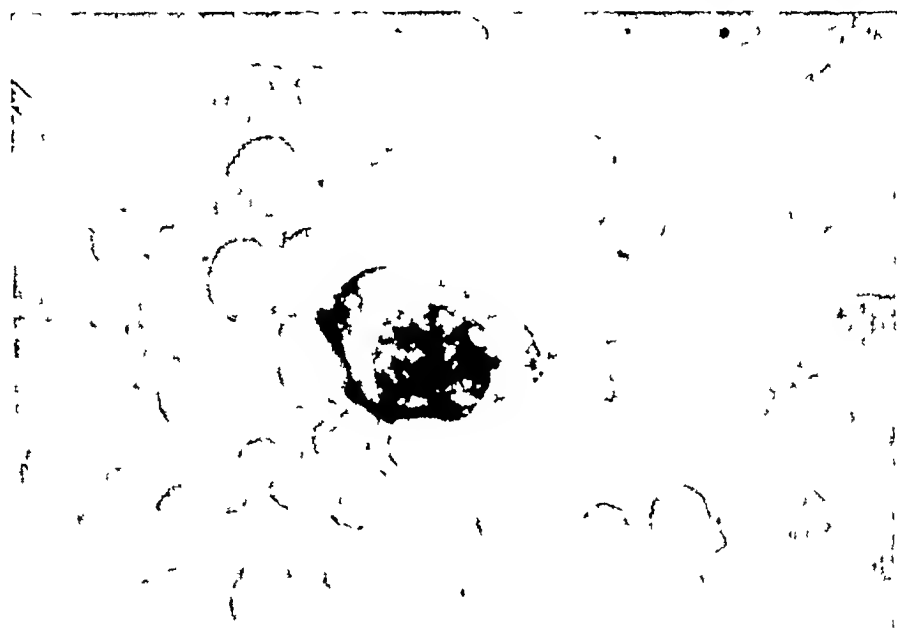


FIG. 14. Here the trend becomes more pronounced. Three definite areas of condensation of chromatin are visible in the nucleus. Note also that the cytoplasm is darker and contains vacuoles ( $\times 1,000$ ).

to the finish and that an imperceptible transition existed between the most reticular cell, the completely characteristic myeloma cell (figs 12 to 16) and the well-differentiated (though atypical) plasma cell. Clinically, this case fitted best a diagnosis of multiple myeloma in a 'plasma cell leukemia' phase. Histologically, the suggestion was almost inescapable that this was a multiple myeloma, that multiple myeloma arose from the reticulo-endothelial cell and that here, in a profound instance of such a disturbance, in which the marrow elements were being liberated into the peripheral blood, the almost completely undifferentiated reticulo-endothelial cell itself was finding its way into the circulation along with its more differentiated counterpart, the myeloma-plasma cell. No other explanation so well satisfies the known facts about multiple myeloma, the experimental studies on the origin of the plasma cell and the very unusual findings in the case mentioned.

## ESTIMATION OF DEGREE OF MALIGNANCY

With the methods used, it was observed that those cases in which there was a marked degree of pleomorphism, often associated with frequent mitoses and notable immaturity, bore the poorest prognosis. Ten cases fell into this category. None of the patients in this group survived longer than twelve months after the onset of symptoms and the mean was 6.3 months. The patient who survived the longest in this group, twelve months, had the most uniform picture, despite the high percentage of myeloma cells present and the reticular, leptochromatic character of the nucleus (fig. 4) in almost all of the cells. The case of the 65 year old man previously described (fig. 2) most strikingly portrays this group. The total duration of this



FIG. 15 More clumping of chromatin is seen here and the cell no longer resembles a reticulo-endothelial cell ( $\times 1,000$ )

patient's disease from the onset of symptoms was one month and his marrow was most anaplastic and pleomorphic.

At the other end of the scale were the 7 cases in which uniform, mature-appearing plasma cells comprised almost the entire number of myeloma cells present, 96 per cent or more. Of the 7 patients, 3 died at the end of forty-two, sixty, and seventy-one months, respectively, and 4 were living when last heard from at the end of twenty-four, twenty-six, eighty-eight and sixty-seven months.

Typical, perhaps, of this group of patients is the case of a woman, 40 years of age, who was first seen in 1941, seven months after the onset of pain in the lower part of her back. She had a palpable liver. Roentgenograms revealed multiple punched-out osteolytic areas in her skull with osteoporosis involving her spinal column. Bence Jones proteinuria was present. Serum proteins were not determined. She was moderately anemic, the concentration of hemoglobin being 8.4 Gm per 100

cc of blood and erythrocytes numbering 3,410,000 per cubic millimeter of blood, and she had mild leukocytosis, the leukocytes numbering 11,300 per cubic millimeter of blood. There was albuminuria, grade 3 (on the basis of 1 to 4, in which 1 designates the mildest and 4 the most severe condition) and a concentration of urea of 38 mg per 100 cc of blood.

The patient's only treatment consisted of orally and parenterally administered calcium salts prescribed by her veterinarian brother. Two years later she wrote that she felt better than she had in ten years and was mowing her own lawn. When heard from in 1945, fifty-eight months after the onset of her illness, she was hospitalized for a pathologic fracture of her left hip. She had had no treatment whatsoever in the preceding two years. She was last heard from in April, 1948,

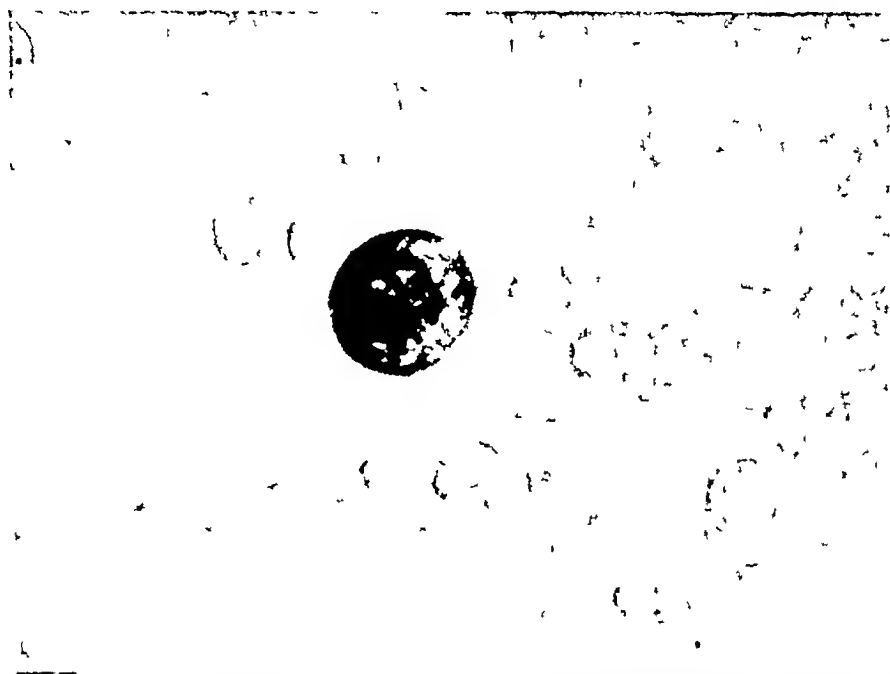


FIG. 16 At this point a moderately well-differentiated myeloma-plasma cell emerges. It will be noted that there has been a gradual loss of cytoplasm, but in each instance the nucleus has tended to remain eccentric ( $\times 1,000$ ).

eighty-eight months after the onset of her illness, and was still feeling fairly well. Ninety-nine per cent of this patient's myeloma-plasma cells were considered to be mature (fig. 1).

Of the remaining patients, 2 could not be traced and 2 were still living. The duration from the onset of symptoms regarded as referable to the disease varied from five to forty-four months. As was anticipated, the trend was toward a longer survival among those patients in whom the greater number of myeloma cells was mature. Of this intermediate group of patients, the following case history is probably most illustrative.

A man, 45 years old, was first seen at the clinic in February, 1945, with a history of anemia and weakness of one and a half years' duration. For the preceding six months he had manifested a marked tendency to bleed from his nose, gums, and

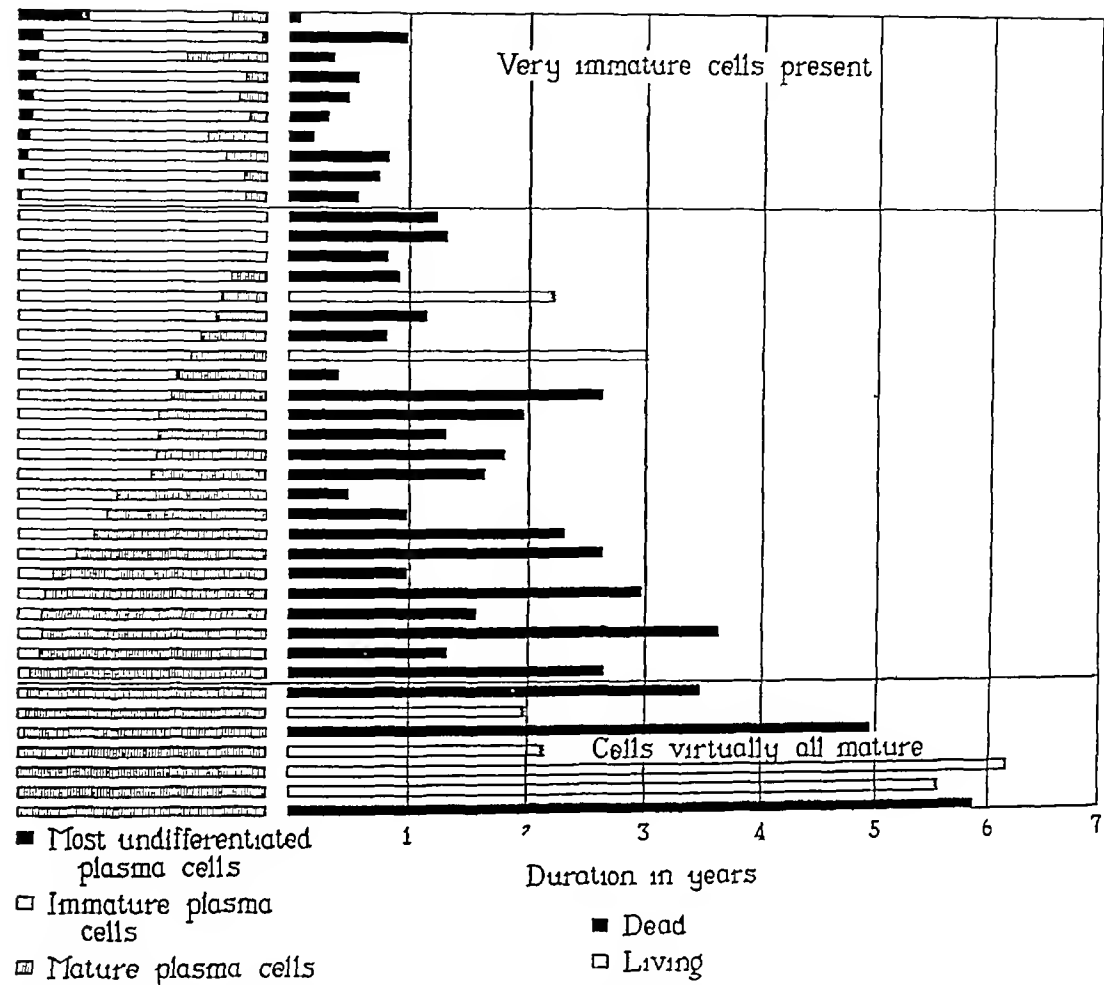


FIG 17 The column at the left represents a differential count of one hundred myeloma cells on each case followed for at least a year. The bars immediately to the right indicate the duration of the disease in the corresponding case from the onset of symptoms.

TABLE 3 —Condition at Last Report. A Summary of the Survivals among those Patients Depicted in Figure 17

Survival from onset, years	Grade 1*		Grade 2		Grade 3	
	Living	Dead	Living	Dead	Living	Dead
0-1			2†	4		9
1-2				11		1
2-3	2		1	5		
3-4		1	1	2		
4-5		1				
5-6	2	1				
Total	4	3	4	22	0	10

\* Graded 1 to 3 on the basis of 1 being the least malignant and 3 being the most malignant  
† Untraced

lacerations of his skin. Osteolytic lesions were demonstrated in his roentgenograms. Bence Jones proteins were found in his urine and his total serum proteins were 11.8 Gm per 100 cc of serum with an albumin-globulin ratio of 1:2.19. The erythrocyte sedimentation rate (modified Westergren method) was 151 mm at the end of an hour. Stem cells were occasionally seen in smears of the peripheral blood and there was excessive rouleaux formation.

The patient had no treatment other than local radium applications for severe epistaxis and died ten months later. Thirty-six per cent of the myeloma cells were of the moderately immature type, and the other 64 per cent were rather well differentiated.

All of the foregoing cases are summarized in table 3, and the duration of the disease in each case is graphically portrayed in figure 17.

It will be observed that several patients of the last-mentioned intermediate group lived only a very short time after the onset of symptoms. Since it is perfectly obvious that the onset of symptoms and the onset of the pathologic process in this disease do not correspond, it was not surprising to note some lack of correlation, and it was in this area that the value of the method used was most limited. It may also be mentioned, though this too, should be apparent, that the discrepancy between the onset of the disease and the onset of the initial symptom is not a constant. However, as will be noted from figure 17, the differential cytologic picture does bear a rather significant relationship to the expected duration of the disease in the group as a whole.

#### SUMMARY AND CONCLUSIONS

Generalizing, it can be said that the pathologic cells seen in smears of the bone marrow in multiple myeloma resemble the plasma cell and vary from the very anaplastic and immature cell to the well-differentiated and almost characteristic plasma cell.

The feature which the "myeloma" cell shares with the plasma cell is the abundant, granular, basophilic cytoplasm which tends to be fragile and undergo the same degenerative changes in each, namely, the formation of Russell bodies and vacuolization. Fairly frequently a perinuclear clear area or Hof is present and the nucleus tends to be eccentrically placed. Cytoplasmic extensions or pseudopodia may also be seen in either case, but they occur more often and more dramatically in instances of multiple myeloma. Multinucleated cells are commonly seen.

In addition, myeloma-plasma cells will often have a large clear nucleolus and a leptochromatic nucleus and will exhibit a tendency to the formation of isolated areas of condensed chromatin. Cytoplasmic extrusions, free cytoplasmic bodies, occasionally complete with Russell bodies and vacuoles are almost universally present.

All cases were of the plasma cell type, there was no exception. In these cases, the myeloma-plasma cell constituted from 2.5 to 96 per cent of the leukocytic elements present. The opinion was expressed that all so-called types of multiple myeloma are merely variations in differentiation of this same cell.

It was noted that anaplasia, hypernucleation and lack of plasma cell predomi-

nance in certain cases were diagnostic pitfalls. Additional evidence was adduced to confirm the reticulo-endothelial origin of the myeloma-plasma cell. It was further observed that certain prognostically valuable information could be gleaned from a careful review of the cytologic characteristics in these cases.

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# UNUSUAL REMISSION AFTER RADIOPHOSPHORUS THERAPY IN A CASE OF "ACUTE PLASMA CELL LEUKEMIA"

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THE DESIGNATION, "acute plasma cell leukemia," has been used in this case for descriptive purposes and is not intended to convey the impression that we conceive of plasma cell leukemia as an independent process apart from the myelomas. Our experience with this disease and that of others have led us to agree with Patek and Castle<sup>1</sup> that plasma cell leukemia merely represents a phase or extension of multiple myeloma. The reasons for this have been stated in detail previously.<sup>2</sup> No attempt will be made at this time to review the literature on this subject. Two recent articles<sup>3, 4</sup> on plasma cell leukemia have brought the literature up to date and any report from us would be incomplete without an analysis of the other cases from the Mayo Clinic which will be attempted in the future. However, it should be mentioned that cases in which the leukemic aspects of the disease predominate are exceedingly unusual (reported in only four instances without roentgenographically evident lesions of bone) and, so far as we know, a remission in such cases has heretofore been unrecorded.

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## REPORT OF A CASE

A 59 year old barber of Italian extraction was admitted to the clinic on Jan. 7, 1946. He gave a history of dizziness, thoracic pain and progressive weakness of two months' duration. Three or four weeks before admission a distressing cough and rapid enlargement of cervical and axillary lymph nodes developed.

At the time of admission the patient was too weak to dress himself, was markedly dyspneic at rest, and was racked by coughing.

On physical examination, pallor and moderate to notable enlargement of the cervical, supraclavicular, axillary, epitrochlear, inguinal, and femoral nodes were observed. Basal rales and hepatosplenomegaly were noted. The liver and spleen were firm and extended below the costal margin about 8 cm. By percussion it was determined that the mediastinum was widened. The blood pressure was 122 mm. of mercury systolic, and 68 mm. diastolic. The pulse rate was 96 beats per minute and the temperature was 98° F.

Special smears of the peripheral blood revealed the presence of 41.5 to 48 per cent of plasma cells (fig. 1). Results of the other laboratory examinations of the blood are given in table 1. The bleeding time (Duke's method) was more than a half hour, and the coagulation time was six minutes. Clot retraction did not occur in twenty-four hours. The sedimentation rate of erythrocytes was 120 mm. in an hour (modified Westergren method), the concentration of serum proteins was 9.6 Gm. per 100 cc. and the albumin-globulin ratio was 1:3.74. The value for serum calcium was 8.1 mg. per 100 cc., for serum phosphorus, 3.6 mg. per 100 cc., and for alkaline phosphatase, 2.5 Bodansky units per 100 cc. of serum.

Urinalysis revealed high grade albuminuria, Bence Jones proteinuria, occasional erythrocytes, and granular and hyaline casts.

Roentgenograms of the patient's skull, ribs, spinal column, pelvis, and right femur revealed no osseous abnormalities. The parenchyma of the lung was considered to be infiltrated diffusely, but the condition could not be distinguished roentgenographically from passive congestion. A biopsy of a cervical lymph node revealed, according to the pathologist, lymphoblastoma containing plasma cells and evidence of involvement of the blood vessels. Atypical myeloma with features of lymphosarcoma and leukemia. Results of examination of material obtained by sternal aspiration were considered to be diagnostic of multiple myeloma (fig. 2 and table 2).

The patient was hospitalized upon admission and remained in the hospital during the following forty-eight days. During the first two weeks he received 500 cc of blood and three small doses of radiophosphorus,  $P^{32}$ . The latter were given almost out of sheer desperation, without the faintest hope of effecting any perceptible improvement.

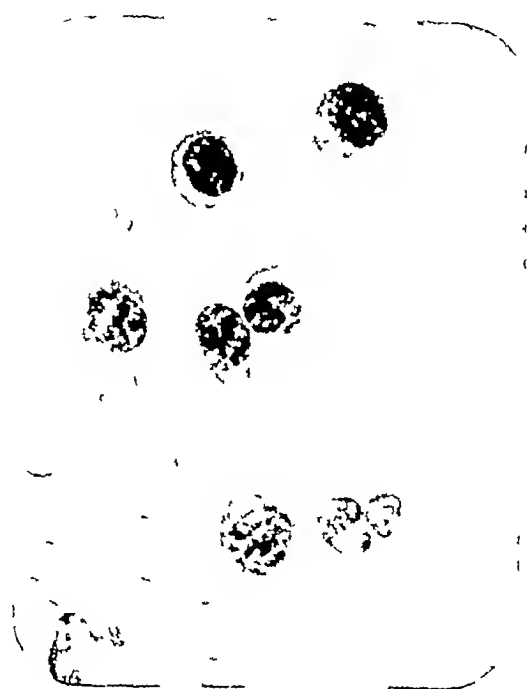


FIG. 1 Smear of peripheral blood in January, 1946. Plasma cell leukocytosis and increased rouleau formation are apparent (Wright's stain,  $\times 640$ ).

TABLE 1—Results of Examination of the Peripheral Blood

	Jan 7, 1946	Jan 23, 1946	Sept 23, 1947
Hemoglobin, Gm per 100 cc of blood	10.9	6.8	14.1
Erythrocytes, per cu mm of blood	3,620,000	2,240,000	4,520,000
Leukocytes, per cu mm of blood	25,600	43,400	5,100
Lymphocytes, per cent	17.5	7.0	48.0
Monocytes, per cent	1.5	4.5	6.0
Neutrophils, per cent	37.0	32.5	45.0
Eosinophils, per cent	1.0	0	1.0
Basophils, per cent	0	0	0
Metamyelocytes, per cent	0	2.5	0
Myelocytes, per cent	0.5	3.5	0
Promyelocytes, per cent	1.0	2.5	0
Plasma cells, per cent	41.5	48.0	0
Platelets, per cu mm of blood		55,000	122,000

The patient's condition grew progressively worse until, at the end of the seventeenth day, it appeared to be in its terminal stage. His temperature was 104 F, pulse rate, 108, and respiratory rate, 28. An indwelling catheter was inserted because he could not void; he was incontinent of feces. A bed sore was developing and blood was oozing from his gums. He was drowsy and deaf (this had appeared within

thirty-six hours, two days previously) and a clear yellow exudate was draining from his left ear. Jewel-clear, pemphigus-like vesicles on his neck, arms, thorax, groins and thighs had appeared and subsided

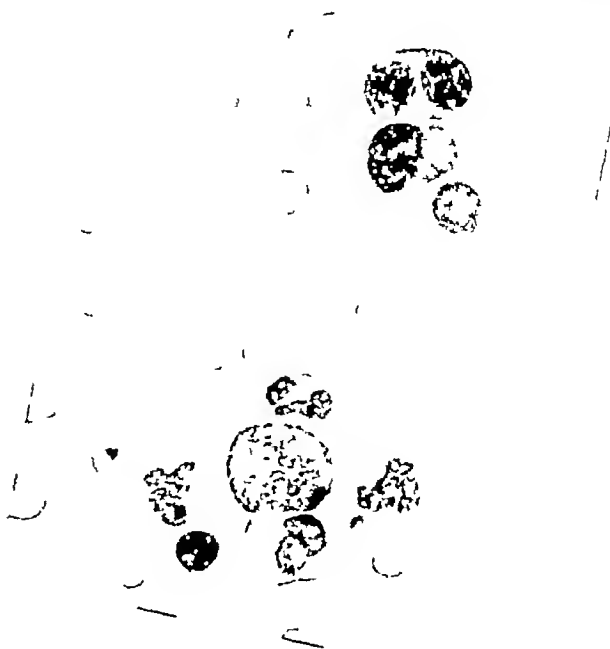


FIG 2 The bone marrow on initial admission shows contrast between the myeloma plasma cells and the lymphocytes. Plasma cells comprise 31.5 per cent of the myeloid elements in this marrow (Wright's stain  $\times 640$ )

TABLE 2—Results of Examination of Sternal Marrow

	Per cent	
	January 1946	September, 1947
Stem cells	1.0	1.5
Leukoblasts	1.0	2.5
Promyelocytes	1.5	4.0
Myelocytes	9.0	6.0
Metamyelocytes	12.0	14.0
Bands	13.0	21.0
Polymorphonuclear cells	5.0	17.0
Eosinophils	0.5	0.5
Basophils	0	0.5
Monocytes	2.5	3.0
Lymphocytes	23.0	28.5
Plasma cells	0	1.5
Myeloma-plasma cells	31.5	0
Nucleated erythrocytes	35.0	22.5

These were the lesions of clinical pemphigus, but were also deemed to be consistent with the terminal manifestations of a disease such as this

The patient lay quietly in bed, with his eyes closed, occasional twitching of his hands and arms was noted and he responded in only a whisper when spoken to. All reflexes were diminished. His abdomen was distended and his mouth was dry. Noisy bubbling râles obscured auscultation. His spleen was markedly enlarged. Examination of the blood was carried out and results are given in table 1. Penicillin, more blood and more radiophosphorus were given in the ensuing few days.

A little more than three weeks after this seemingly terminal episode the cervical nodes were estimated to be 50 per cent smaller, the lungs seemed clearer, and the spleen was about 25 per cent smaller. The number of plasma cells in the peripheral blood was markedly reduced, and the leukocyte count had dropped to 6,600. However, the erythrocytes numbered only 3,100,000 per cubic millimeter and the value for hemoglobin was only 7.1 Gm per 100 cc.

On Feb. 23, forty-eight days after his admission to the clinic, it was felt that the patient had recovered sufficiently to go home.

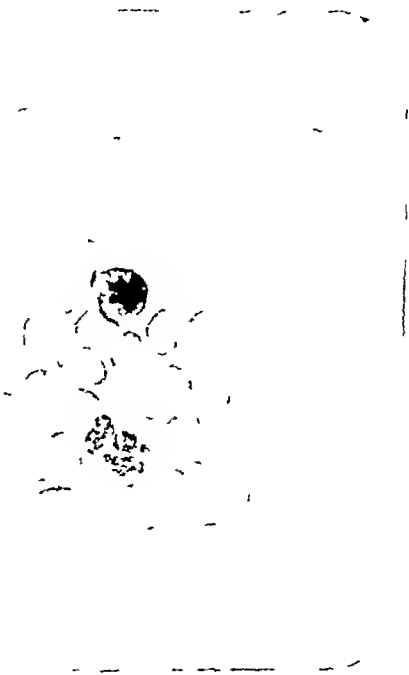


FIG. 3. Smear of the peripheral blood in September, 1947. It is apparent that there are no longer leukocytosis, rouleau formation or plasma cells. The lymphocytes and neutrophils are present in approximately equal numbers (Wright's stain,  $\times 640$ ).

In the course of his stay at the clinic, he had received 1,770,000 units of penicillin, 3,000 cc of whole blood and biweekly intravenous injections of radiophosphorus,  $P^{32}$ . The total dose of radiophosphorus was 6,135 microcuries.

The patient returned for a recheck on Sept. 22, 1947. The following information was obtained about the interval before his return. He had remained in a hospital elsewhere for three more weeks after his dismissal here and then gradually had become ambulatory. He had received 500 cc of whole blood twice; the second transfusion was in April, 1946. Gradually his strength returned, his thoracic pain subsided, and by Oct. 1946, he had returned to his work on a part-time basis. He had worked daily until the time of his second visit to the clinic and he stated that he was continuing to feel stronger and stronger.

At that time his color was excellent. Lymph nodes, liver and spleen were not palpable. His blood pressure was 130 mm of mercury systolic, and 70 mm diastolic, and results of general physical examination were completely satisfactory.

Also at that time extensive examination of smears of the peripheral blood failed to reveal any plasma cells, merely mild lymphocytosis (fig. 3). Results of some of the laboratory examinations are given in

tables 1 and 2. The value for serum protein was 6.9 Gm. per 100 cc. and the albumin-globulin ratio was 1.9:1. The erythrocyte sedimentation rate was 35 mm. in 1 hour (modified Westergren method). Urinalysis was carried out twice and results were entirely negative. Roentgenograms of his skull, ribs and right shoulder showed no osseous abnormalities and the roentgenogram of his thorax did not reveal any abnormal condition.



FIG. 4. The bone marrow at the time of the patient's last admission shows an essentially normal spread of cells with active erythropoiesis; 1.5 per cent of normal appearing plasma cells are present (Wright's stain,  $\times 640$ ).

TABLE 3—Results of Various Examinations

	January, 1916	September, 1917
Hemoglobin, Gm. per 100 cc. of blood	6.2*	14.1
Erythrocytes, per cu. mm. of blood	2,050,000*	4,520,000
Leukocytes, per cu. mm. of blood	43,400*	5,100
Plasma cells, per cent	48*	0
Sedimentation rate, millimeters in one hour	120	35
Blood urea, mg. per 100 cc.	54	26
Serum protein, Gm. per 100 cc.	9.6	6.9
Albumin-globulin ratio	1.374	1.91
Serum calcium, mg. per 100 cc.	8.1	9.6
Serum phosphorus, mg. per 100 cc.	3.6	2.9
Albuminuria, grade†	3	0
Bence Jones proteinuria	Present	Absent
Roentgenologic examination of bone	Negative	Negative
Examination of sternal marrow	Diagnostic	Not diagnostic

\* These are the most extreme values found in the course of the patient's first visit.

† On a grading basis of 1 to 4 in which 1 represents the least and 4 the most severe condition.



Examination of the sternal marrow (fig 4 and table 2) revealed no evidence of his disease. As a matter of fact, no evidence was found in the course of his whole re-examination. The laboratory findings obtained in the course of his two visits to the clinic are given in table 3 for comparison.

#### COMMENT

We were at no time prepared for such a startling and unprecedented turn of events. Remission, spontaneous or induced, has not been recorded previously to our knowledge, hence, despite the consistently discouraging results from the use of radiophosphorus in multiple myeloma generally, its use in this case is brought into sharp focus. Its possible value in similar instances is suggested and it is hoped that further experience with radiophosphorus in the treatment of this disease will properly define its usefulness as a therapeutic agent.

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# BARTONELLA BACILLIFORMIS ANEMIA (OROYA FEVER)

## A STUDY OF THIRTY CASES

By WILLIAM E. RICKETTS, M.D.

### INTRODUCTION

A SEVERE anemia was noticed in workers stationed in certain localized areas along the deep river valleys in 1870 during the construction of a railroad to Oroya, a Peruvian city located in the Andes. This febrile anemia, called "Oroya fever," was thought to be a new disease. In 1885, Daniel A. Carrión, a Peruvian medical student, developed a fatal anemia with the characteristics of Oroya fever, twenty-one days after self inoculation with blood obtained from a verrucous node of the skin of a patient with *Verruga peruviana*. This proved that the anemia was an initial stage of the latter. Thus the name of Carrión's disease<sup>1</sup> has honored the Peruvian martyr.

Since the etiologic agent of the disease was not known during the last century and part of the first decade of the present, excellent clinical descriptions of the disease<sup>2</sup> undoubtedly included cases complicated by intercurrent infections. A pleomorphic bacilliform organism was discovered by the Peruvian physician, Alberto Barton, in 1909,<sup>3</sup> to be the pathogenic agent of the disease.

The purpose of this paper is to analyze the clinical and hematologic characteristics of a series of 30 cases of anemia with *Bartonella bacilliformis* present in the circulating erythrocytes, an entity herein referred to as *Bartonella bacilliformis* anemia. This study was made in Lima, Peru, from 1938 to 1943.

### BARTONELLA BACILLIFORMIS ANEMIA

*Bartonella bacilliformis* anemia (Oroya fever) is a febrile hemolytic anemia with *Bartonella* parasitizing the erythrocytes. This is an infrequent clinical manifestation of the invasive stage of Carrión's disease. It is interesting to notice that in areas where the disease is endemic and where most of the natives have had the disease, few are thought to have had the anemia. At present, however, there is no statistical report available from these endemic areas indicating the exact incidence of *Bartonella bacilliformis* anemia in Carrión's disease. "Oroya fever," Grave fever of Carrión<sup>2</sup> are synonyms for this anemia, the most used, Oroya fever, is inappropriate as the *Bartonella* infection does not occur in the Peruvian city of Oroya.

It has distinguishing clinical and hematologic features and develops so rapidly that it can be compared only with the anemia occurring in an acute hemorrhage. This anemia occurs irrespective of age, sex or race. Before the onset of the disease the patients of these series were all healthy, well nourished, young individuals with nothing suggesting a deficient individual resistance.

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The physical findings in the patients with severe anemia are rather dramatic and almost pathognomonic. They are deeply apathetic and have a peculiar discoloration of the skin and sclerae due to the combination of slight icterus with very severe anemia. The conjunctivae and the matrix of the nails are almost colorless and the ears almost transparent. The cardiovascular signs consist of tachycardia, soft hemic murmurs of varied intensity heard over the entire precordium, synchronously there are suprasternal, epigastric, carotid pulsations, the blood pressure is moderately hypotensive and occasionally there is peripheral collapse. Dyspnea is very unusual in anemic cases without intercurrent infections, even with erythrocyte counts below one million, contradicting previous reports,<sup>2 15</sup> in cases with some intercurrent infection, especially salmonellosis, it is a constant finding.<sup>13 14</sup> Cough and expectoration with rhonchi and rales on physical examination early in the disease may simulate a primary upper respiratory infection. Cheyne-Stokes respiration has been described.<sup>5</sup> This occurred only in the complicated cases of the present series. Headache, vertigo, restlessness and drowsiness, tinnitus, insomnia and occasionally angina pectoris are symptoms dependent on the intensity of the anemia. Patients often complain of a feeling of transmitted cardiac beating to the head and ears. "The patient hears his murmurs," said Odriozola.<sup>2</sup>

Thirst and anorexia are common features. The tongue is usually dry with a coffee-brown coating. In the early stages of the anemia, profuse sweating may occur but later the skin becomes very dry, a significant point in the differentiation from malaria. There is a generalized lymphadenopathy with nontender lymph nodes, of 5 to 15 mm, without paradenitis. Enlarged lymph nodes occurred in all except three fatal cases. Enlargement of the spleen which has been described as a constant finding,<sup>2 5 6</sup> appeared only in the cases with intercurrent infections of this series, thus confirming a previous report.<sup>7</sup> The liver was moderately enlarged in only 2 of the 15 noncomplicated cases.

Observations on the course and type of fever in the past have been varied and even contradictory.<sup>8 5 9 2 6 10-13</sup> Often after a variable onset with or without chills, the temperature fluctuates between 37.5 and 38.5 C, but it may be higher or lower, and occasionally patients with severe anemia run an almost afebrile course. The severity of the anemia does not parallel the intensity of the fever. Intermittent fever<sup>2 9</sup> is usually due to concurrent malaria. Odriozola<sup>2</sup> observed that in many cases there was later a marked elevation of temperature, which he interpreted as prodromal to a second outbreak or "hyperthermic" course. This elevation of temperature has been found<sup>13 14</sup> to be associated with intercurrent infection, usually due to *Salmonellae* organisms.

Petechial hemorrhages in the skin are not uncommon. This is mentioned in the first descriptions of the disease and occurred in the martyr Carrión himself.<sup>1</sup> The pinpoint hemorrhages were originally thought to be produced by the beginning eruption of verrucae<sup>2</sup> and the epistaxis supposedly produced by verrucae in the mucosa of the nose. This explanation is not satisfactory, for most of the cases with epistaxis occur during the initial stage of the disease when verrucae are not seen. Malo<sup>19</sup> first made this differentiation between the petechiae and the pinpoint verrucae in the skin. The author demonstrated in a previous study that cases with

purpura have a constant thrombocytopenia.<sup>17</sup> Hemorrhage may vary from petechial spots to ecchymoses, epistaxis, gingival hemorrhage, hematemesis or melena.<sup>17 18 19 20</sup> In Case 1, epistaxis was so intense as to endanger the life of the patient, in Case 4, purpura was probably the cause of death, and in Case 5, which was fatal, large hematomas occurred. Clouding of the sensorium and delirium are rather common.<sup>20 21</sup> In most anemic crises mental symptoms, such as irritability and insomnia, were of secondary importance. However, in other cases, the mental symptoms were so prominent as to constitute a real psychosis. Piccola<sup>22</sup> first described these psychotic episodes and considered them transitory and related to the course of the disease. Alterations of the memory and consciousness were common. In the present series of 30 cases with bartonella anemia, 8 had meningococcic infection with Case 3, these appeared as the end stage of a very virulent bartonella infection with pronounced anemia. In all other cases, these symptoms were present during the crisis of the anemia, as described in Cases 3, 5 and 6. Pain in the bones, joints and muscles along with cramps may occur, but without the constancy and intensity which are found later in the pre-ruptive stage of the disease.

#### HEMATOLOGIC FINDINGS

As early as 1898, Tamayo<sup>23</sup> and Herccles<sup>24</sup> described a fall in the erythrocyte count and in the hemoglobin values together with anisocytosis and poikilocytosis. Since then many hematologic studies have been made,<sup>25 26 27</sup> including a very complete monograph by Hurtado et al.<sup>18</sup> The hematologic findings in 14 cases in this series with apparently uncomplicated bartonella anemia are included in tables 1A and 1B. Hematologically, this anemia is characterized by a sharp drop in the erythrocyte count, which may be so intense as to reach 0.5 million red cells, as is illustrated by Cases 1, 3, 5 and 7. This drop occurs in the first two to four weeks of illness. In Case 3, the erythrocyte count on the day of death, the tenth day of symptoms, was 0.625 million per cu. mm. The anemia is macrocytic and usually hypochromic. Case 1 had a mean corpuscular volume of 198 cubic microns, with a mean corpuscular hemoglobin concentration of 29.8 per cent. The peripheral blood shows marked regenerative activity of both erythroid and myeloid series. The number of nucleated red cells may be exceedingly high. Case 1 had 20 erythroblasts and 461 normoblasts per 100 leukocytes, or 37,164 normoblasts per cubic millimeter. The hematocrit decreased proportionally to the fall in the erythrocytes and in Case 3 reached 9.81 per cent. The lowest hemoglobin value, 1.77 Gm. per cent (using the Evelyn colorimeter) in this series of cases occurred in Case 2 who recovered. Frequently, the erythrocytes show light refractile basophil granulations, nuclear particles, Howell Jolly bodies, polychromasia, poikilocytosis and anisocytosis. Reticulocytes may increase to 50 per cent. The pathognomonic sign of the disease in the erythrocyte is the bartonella bacilliformis. This organism is very polymorphous. In Giemsa staining, it appears as red-violet rods situated on the red cells. The "bacilliform" bodies may be as numerous as 20 on each erythrocyte in heavy infections (fig. 1). The length of the organism varies from 1 micron to 3 microns by 0.25 micron to 0.50 micron. They are dis-

TABLE 1A—*Bartonella Bacilliformis* Anemia Uncomplicated Cases

Case	Date	Erythrocytes/cu m	Reticulocytes %	Hematocrit cc %	Hemoglobin Gms per 100 cc	Mean Corpuscular Vol (cubic micron)	Mean Corp Hemoglobin (micromicogram)	Mean Corp Hemoglobin (gm)	Leukocytes per cu m	Polymorphonuclear Neutro- phils per cu m	Myelocytes	Metamyelocytes	Filamented Forms	Segmented Cells	Eosinophiles	Basophiles	Monocytes	Lymphocytes	Krythroblasts per 100 Leukocytes	Normoblasts per 100 Leukocytes	Anysocytosis	Polikilocytosis	Polichromasia	Bartonella	Outcome	
X H 1	7 21 38	57	6	11.9	3.3	198.1	57	29.8	7 600	63	5	7	16	35	1	0	0	4	32	2	194	++	++	++	++	
	7 22 38	61	12.7	11.4	3.4	184	55	29.8	10 000	76	5	19	25	41	0	0	5	19	2	241	++	++	++	++		
	7 23 38	55							6 900	76	2	17	17	41	0	0	4	20	1	361	++	++	++	++		
	7 24 38	55							7 800	75	0	17	18	44	0	0	4	20	1	361	++	++	++	++		
	7 26 38	70							12 200	81	0	17	25	44	0	0	4	18	0	1	++	++	++	++		
	7 28 38	80	27	11.2	3.4	158	47	30.3	5 560	78	0	17	27	44	1	0	4	18	0	1	++	++	++	++		
	7 29 38	91							5 700	74	0	17	25	44	1	0	4	17	0	1	++	++	++	++		
	7 30 38	87							9 100	82	0	17	25	44	1	0	4	17	0	1	++	++	++	++		
	7 31 38	78							11 400	80	0	17	25	44	1	0	4	17	0	1	++	++	++	++		
	8 2 38	83								8 670	69	0	17	25	44	1	0	4	15	0	1	++	++	++	++	
V C H 2	8 4 38	1 03	9.3	21.1	6.2	125	37	24.4	5 760	46	0	17	14	32	2	0	4	33	0	0	++	++	++	++		
	8 9 38	1 68							10 400	49	0	17	18	44	2	0	4	33	0	0	++	++	++	++		
	8 13 38	2 41							7 000	39	0	17	11	28	2	0	4	33	0	0	++	++	++	++		
	8 25 38	3 64	1	35.6	12.2	97.8	33	34.3	6 200	32	0	17	8	26	2	0	4	33	0	0	++	++	++	++		
	8 30 38	3 90	1	36.2	12.4	89	30	34.1	7 700	37	0	17	11	26	2	0	4	33	0	0	++	++	++	++		
	9 11 38	4 09	3	39.1	13.8	81	29	35.3	6 120	42	0	17	9	33	5	0	4	33	0	0	++	++	++	++		
	10 11 38	4 08							5 240	40	0	17	9	33	5	0	4	33	0	0	++	++	++	++		
	11 15 41	78	41	11.7	1.75	150	22	14.9	13 060	58	2	5	15	36	0	0	4	42	0	47	++	++	++	++		
	11 18 41	1 12							5 540	72	0	1	20	31	0	0	4	42	0	47	++	++	++	++		
	11 21 41	1 41	24	21.9	6.05	126	34	27.6	3 550	52	1	5	20	34	0	0	4	42	0	47	++	++	++	++		
R H # 3	11 24 41	2 44	12	22.6	7	93	29	30.8	7 980	68	0	5	19	48	0	0	4	42	0	47	++	++	++	++		
	11 28 41	3 08	1.4	28.3	10	83.5	26	30	6 320	48	0	5	5	25	0	0	4	42	0	47	++	++	++	++		
	12 2 21	3 08	1.4	33.24	12.5	83.5	26	30	7 880	44	0	5	5	25	0	0	4	42	0	47	++	++	++	++		
	12 9 41	3 99	0	39.29	12.5	83.5	26	30	6 990	46	5	5	5	25	0	0	4	42	0	47	++	++	++	++		
	12 24 41	4 58	0						35 000	46	5	5	5	25	0	0	4	42	0	47	++	++	++	++		
	2 2 42	6 9	0																			++	++	++	++	
	7 8 41	65	20	9.81	2.4	198	37	24.2	35 000	46	5	5	5	25	11	0	0	4	54	2	64	++	++	++	++	
	5 16 42	97							3500	56	0	10	10	10	36	0	0	2	42	1	2	++	++	++	++	
	5 13 39	76							6 990	78	7	13	10	28	48	6	0	6	16	1	100	++	++	++	++	
	5 14 39	87							3 350	84	1	10	28	36	4	0	2	2	10	0	2	++	++	++	++	
A B # 4	5 16 39	83							9 180	75	2	13	36	36	4	1	8	8	10	0	26	++	++	++	++	
	5 18 39	86							10 800	72	0	1	36	36	4	1	8	8	10	0	26	++	++	++	++	
	5 22 39	44							12 800	72	2	9	27	36	4	1	8	8	10	0	26	++	++	++	++	
	10 20 39	1.8	8						6080	40	0	3	27	36	4	1	8	8	10	0	26	++	++	++	++	
	10 23 39	2.4	5.2	26.1	7.8	108	45		12 100	39	0	3	27	36	4	1	8	8	10	0	26	++	++	++	++	
J H # 5	10 26 39	2.28	9.3						10 700	56	1	3	28	36	4	1	8	8	10	0	26	++	++	++	++	
	10 28 39	5.41	12.0	39.2	12.5	115	37		12 400	57	1	3	28	36	4	1	8	8	10	0	26	++	++	++	++	
	10 28 39	5.41	12.0	39.2	12.5	115	37		12 400	57	1	3	28	36	4	1	8	8	10	0	26	++	++	++	++	

Chart on this page is per cent myelocytes per cent

Start cells 100 per cent myelocyte range

Case	Date	Erythrocytes/cu m.m.	Reticulocytes %	Hematocrit c.c. %	Hemoglobin Osm per 100 c.c.	Mean Corpuscular Vol (c cubic microns)	Mean Corp Hemoglobin (microcristogram)	Mean Corp Hemoglobin cont.	Leukocytes per cu m.m.	Polymorphonuclear Neutro phils per cu m.m.	Neutrophils	Monocytes	Segmented Cells	Reticulocytes	Platelet Form	Neutrophils	Monocytes	Lymphocytes	Erythrocytes & Normoblasts per 100 leukocytes	Amyeloblasts	Polychromatic	Polychromatic	Maturation
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bartonella	2/23/39	2,42	32	107	34	32	10,200	76	0	0	0	0	0	0	0	0	0						

[illegible]



FIG. 1

*Case 3* Massive parasitism of erythrocytes by *Bartonella bacilliformis* in the peripheral blood. Bartonellae are present on the red cells, and outside the cells. Two erythroblasts and two myeloblasts are also seen.  $\times 2200$  Giemsa stain.

tributed in rods in a Y, V, or chain appearance. They may be curved and may show polar enlargement.<sup>4</sup> These organisms are well stained by the May-Grunwald-

Giemsa method or by any of the modifications of the Romanowski method (fig 1). The relation of the bartonellae to the red cells has been a controversial point. Modern studies<sup>39</sup> tend to indicate that the bartonellae are not located within the red cells, but are rather superimposed on them.

While leukocytosis has been described in this anemia,<sup>23, 35-37</sup> others have reported a normal leukocytic count<sup>15</sup> or even a tendency to a leukopenia.<sup>17, 33</sup> It seems from this study that the leukocyte count varies in each case, and in the same patient during the course of the disease. Slight leukocytosis is not uncommon, but marked leukocytosis in cases without intercurrent infections is extremely rare. This occurred, however, in Case 12 (table 1B) with a leukocyte count of

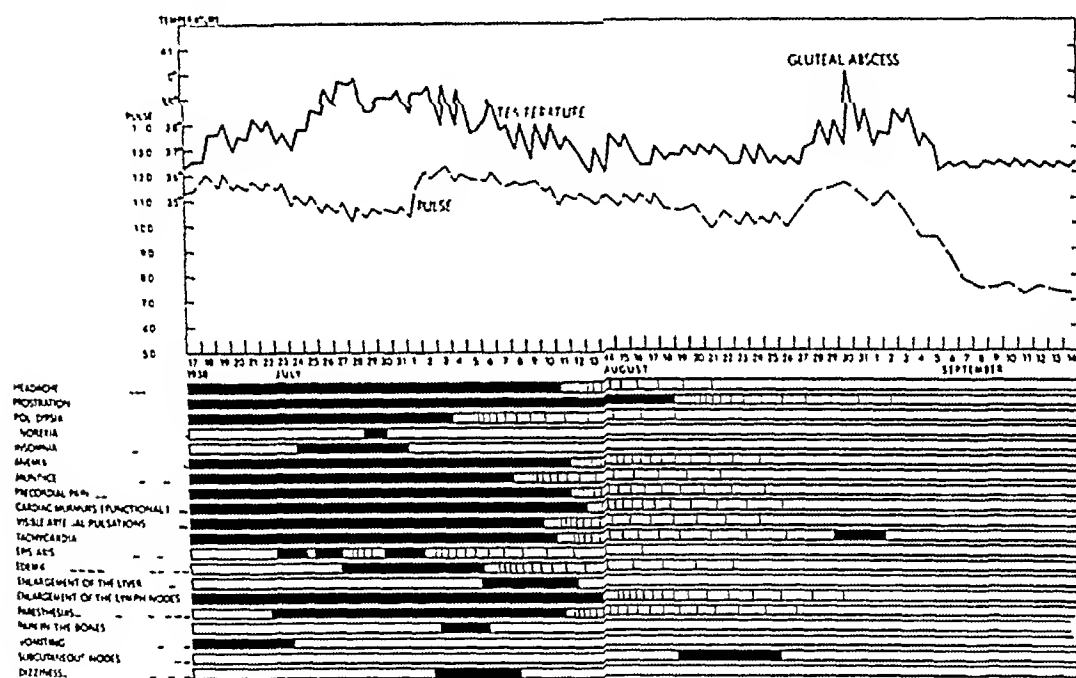


FIG. 2.

*Case 1.* Parallelism between the symptoms and the temperature and pulse in a case of *Bartonella bacilliformis* anemia. The solid blocks represent a marked severity of the symptoms, the lines indicate a lesser degree of severity (the greater the distance between lines, the less marked are the complaints), the empty blocks represent absence of symptoms.

30,160. A very frequent finding is a shift to the left characterized by the presence of myeloblasts, myelocytes, and metamyelocytes. Eosinophilia existed in only one case where *ascaris lumbricoides* was demonstrated in the stools. The leukocyte count appears to be of value as an index to the course of the bartonella infection and in detecting intercurrent infections.

Cases 1, 2 and 3 which follow illustrate severe bartonella bacilliformis anemia.

*Case 1 (Figs. 2, 3 and 4)*

A V, a 17 year old Indian laborer, after living for six weeks in an area where Carrion's disease is endemic, developed in the first few days of July, 1938, a general feeling of malaise, fever, profuse sweating, pains in the bones of the hands and feet, shortness of breath on exertion and precordial pain. He was admitted to the hospital in Lima on July 17, 1938, in good general nutrition, appearing anemic and



slightly icteric. There were abnormally intense carotid arterial pulsations synchronous with the pulse, and functional hemic murmurs over all areas of the heart, the blood pressure was 110/50, the pulse 120 per minute, and the respirations were accelerated and very shallow. The patient rapidly became confused, had psychomotor excitability, the anemia increased and the fever continued between 37 and 38.2 C. He complained of occipital headache, precordial oppression and insomnia, epistaxis occurred on several occasions. On July 21, 1938, the erythrocyte count totaled only 0.575 million per cu mm, the hemoglobin was 3.3 grams per cent and the leukocytes 7,600. There was a macrocytosis of 198 cubic microns and 461 normoblasts per 100 leukocytes. The red cells showed marked anisocytosis, poikilocytosis and polychromasia with many bartonella bacilliformis. The Wassermann and Kahn reactions were strongly positive. The symptoms continued unchanged until July 28. On that day, no bartonellae

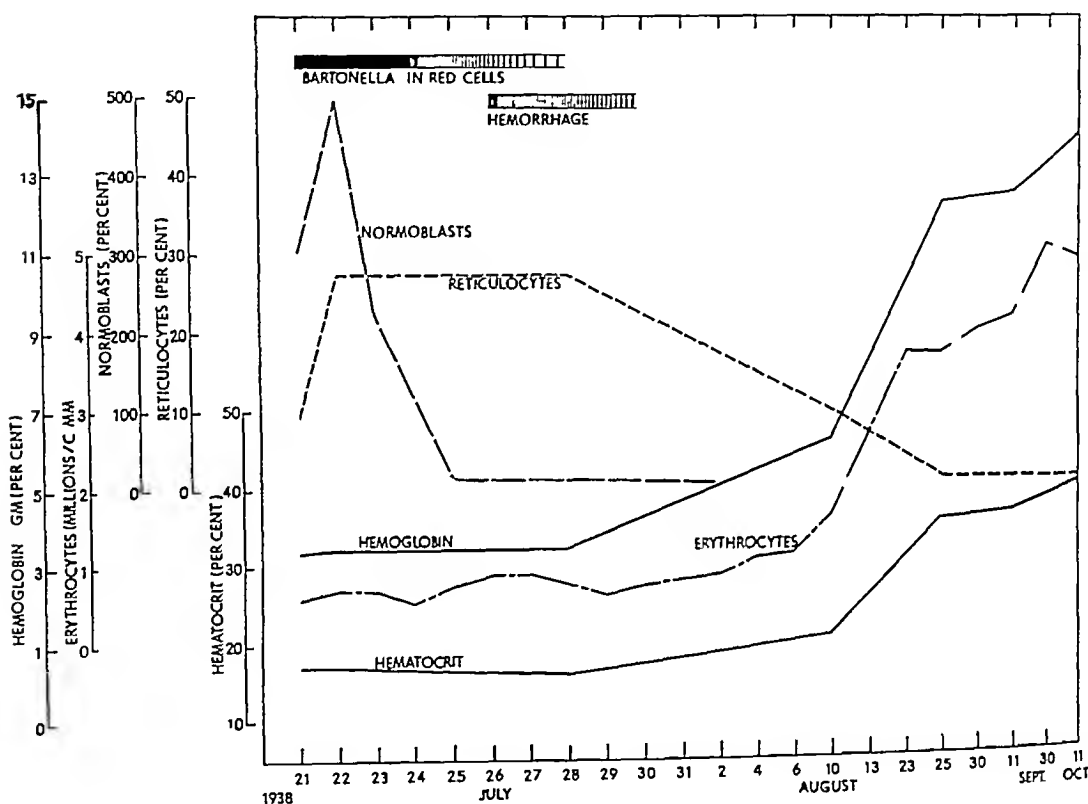


FIG 3

*Case 1* Critical and recovery phases of *Bartonella bacilliformis* anemia, showing the marked increase of the red cells, hemoglobin and hematocrit after the disappearance of the *Bartonella bacilliformis*.

At the top, in the bars representing *Bartonella* in red cells, and hemorrhage, solid black indicates heavy concentration of *Bartonella bacilliformis*, gradually widening spaces indicate progressive diminishing number of *Bartonella bacilliformis*.

were present in the erythrocytes. On July 29, the general condition of the patient became worse with insomnia and delirium during the night, he had severe epistaxis with loss of approximately 500 cc of blood, the platelets numbered 80,000, the red cells decreased from 0.82 to 0.62 million. The patient remained apathetic in bed and slight edema of the eyelids was noticeable for the first time. The temperature rose to 39 C. Blood cultures were negative for bacteria other than *Bartonella bacilliformis*. During the first week of August, his general condition improved strikingly despite the fever. The number of red cells increased considerably without any therapy, the symptoms faded and the patient developed a ravenous appetite. On October 11, 1938, before leaving the hospital, the erythrocyte count was 4.54 million/cu mm, the hemoglobin 13.8 grams per cent, the hematocrit 39.1 per cent. The mean corpuscular volume was 81, the leukocytes 5,240. Wassermann and Kahn reactions found strongly positive on admission were considered false. These tests later were repeatedly negative.

This case illustrates a severe bartonella anemia with nearly a half million erythrocytes/cu mm, an unusual intensive erythrocytic regeneration and an exceptional macrocytosis. Nasal bleeding coexisted with thrombocytopenia. Despite a prompt recovery an unexplained febrile episode followed the disappearance of bartonellae from the erythrocytes.

#### Case 2 (Figs 5 and 6)

V. C. S., a 21 year old Peruvian male, on April 19, 1941, was sent for ten days to an area where Carrion's disease and malaria are endemic. On July 28, he developed malaria which disappeared after three days of intense antimalaria treatment.

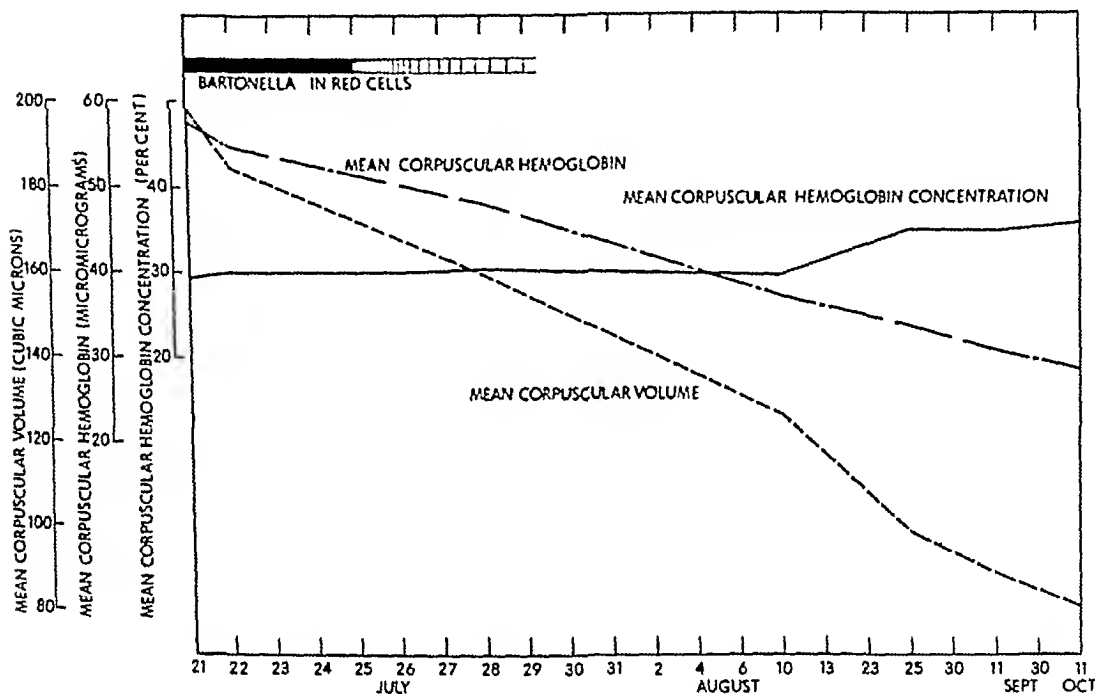


FIG 4

Case 1. Mean corpuscular values during the recovery phase of *Bartonella bacilliformis* anemia showing a striking fall in the mean corpuscular volume.

At the top, in the bar representing *Bartonella* in red cells, solid black indicates heavy concentration of *Bartonella bacilliformis*, gradually widening spaces indicate progressive diminishing number of *Bartonella bacilliformis*.

On November 1, he again developed fever and malaise, preceded by a single chill. Mental stupor, marked psychomotor excitability and delirium soon developed, the fever being slight and continuous. On admission (November 8, 1941), the patient was mentally confused and in such a serious condition that it was thought he would die shortly. There was a very severe anemia and jaundice, functional murmurs were present all over the precordium, the blood pressure was within normal limits, the pulse was 120 and the respirations were regular, 16 per minute. Only a few lymph nodes less than 1 cm in diameter were palpated in both axillae. The urine was normal. The van den Bergh reaction for bilirubin was of an indirect type, and the Wassermann and Kahn reactions were negative. The red count was 0.785 millions/cu mm with 46 per cent reticulocytes, hemoglobin 1.75 grams (Evelyn photocolormeter), hematocrit 11.7 per cent, the mean corpuscular volume was 149.9 cubic microns, and icteric index 15. The white blood count was 13,060, with 58 polymorphonuclear neutrophils (2 myelocytes, 5 metamyelocytes, 15 filamented and 36 segmented cells) and 42 lymphocytes. There were 47 normoblasts per 100 leukocytes. Pronounced anisocytosis, poikilocytosis and polichromasia were found. A very small

percentage of erythrocytes contained coccoid bartonellae After a few days, the existing bartonellae disappeared, the number of erythrocytes increased rapidly, the mean corpuscular values became normal, the slight jaundice cleared, the mental symptoms improved and the fever disappeared In the last two weeks of December and the first week of January, he had transitory pains in the epiphysis of the long bones, in the short bones of the hands and feet and in the kness He had paresthesias, the sensation of small worms in the skin Two red spots not larger than a pinpoint were noticed on the skin, corresponding probably to verrugae in formation No anemia was recorded the day the patient left the hospital, January 22, 1942

This case illustrates a very prompt recovery after a severe bartonella bacilliformis anemia

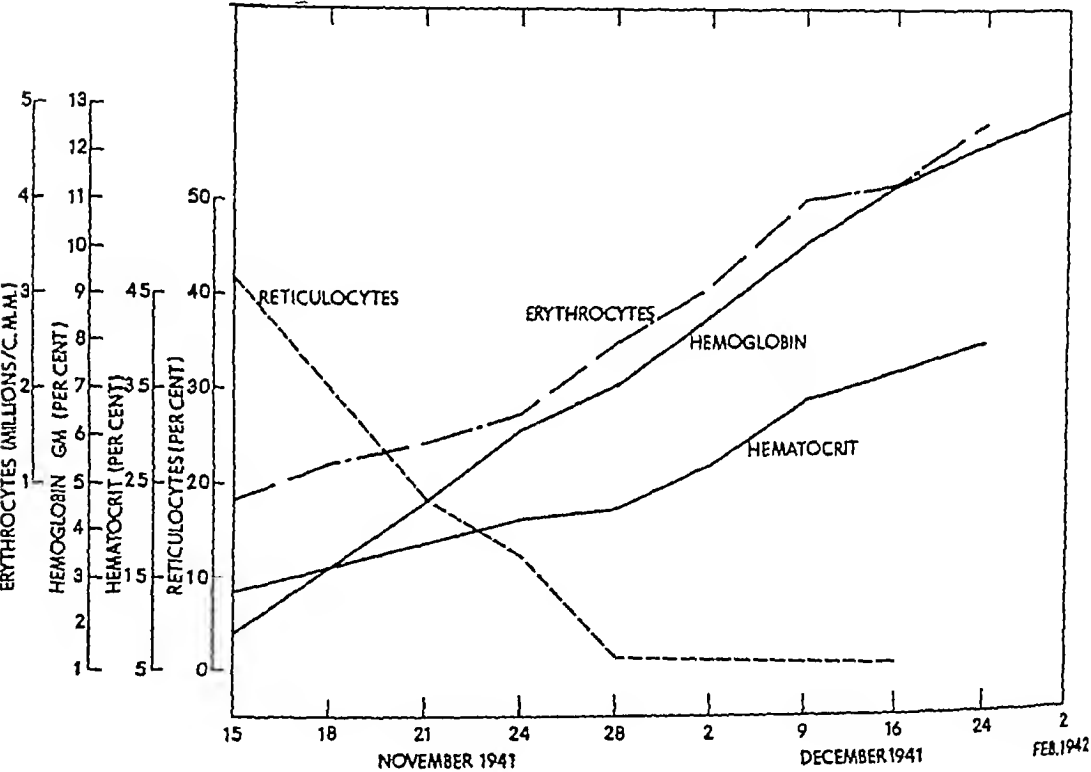


FIG 5

Case 2. Recovery phase of Bartonella bacilliformis anemia

Case 3

R. H., a 7 year old native Peruvian boy, came from a town where Carrion's disease is endemic On May 28, 1941, he developed malaise and lack of appetite, however, he continued going to school until June 1 His mother noticed that he had fever, had vomited several times and had become anemic and slightly jaundiced Two days before admission he became restless and delirious In view of the seriousness of this condition, he was brought to the Children's Hospital in Lima on June 8, 1941 Physical examination showed a very intense anemia The sclerae of the eyes were slightly icteric, the pupils were dilated, but reacted well to light He had meningeal signs, rigidity of the neck, maxillary trismus, loss of tendinous reflexes, and bilaterally absent Babinski reflexes The lymph nodes in the neck were slightly enlarged, the liver was palpated 3.5 cm below the costal margin in the right mid-clavicular line, there were marked carotid pulsations in the neck, together with functional murmurs all over the precordium The urine was normal On July 8, 1941, the erythrocyte count was 0.625 million, the leukocytes 35,000,

tetuculocytes 20.2 per cent, hemoglobin 2.4 grms per cent ( Evelyn photocolormeter), hematocrit 9.81, icterus index 20, mean corpuscular volume 158.55 cubic microns, mean corpuscular hemoglobin 38.40 micromicrograms, mean corpuscular hemoglobin conc. 24.22 per cent. The differential count was polymorphonuclear neutrophils 44 (5 myelocytes, 5 metamyelocytes, 25 filamented forms and 11 segmented cells), lymphocytes 54 per cent, promyelocytes 2. There were 1 erythroblast and 64 normoblasts per cent. There were pronounced anisocytosis, poikilocytosis and polichromatosis. Ninety-six per cent of erythrocytes contained bartonella bacilliformis and as many as 19 bartonellae were present in each red cell (fig. 1). The patient died on July 8, 1941, and no autopsy could be obtained.

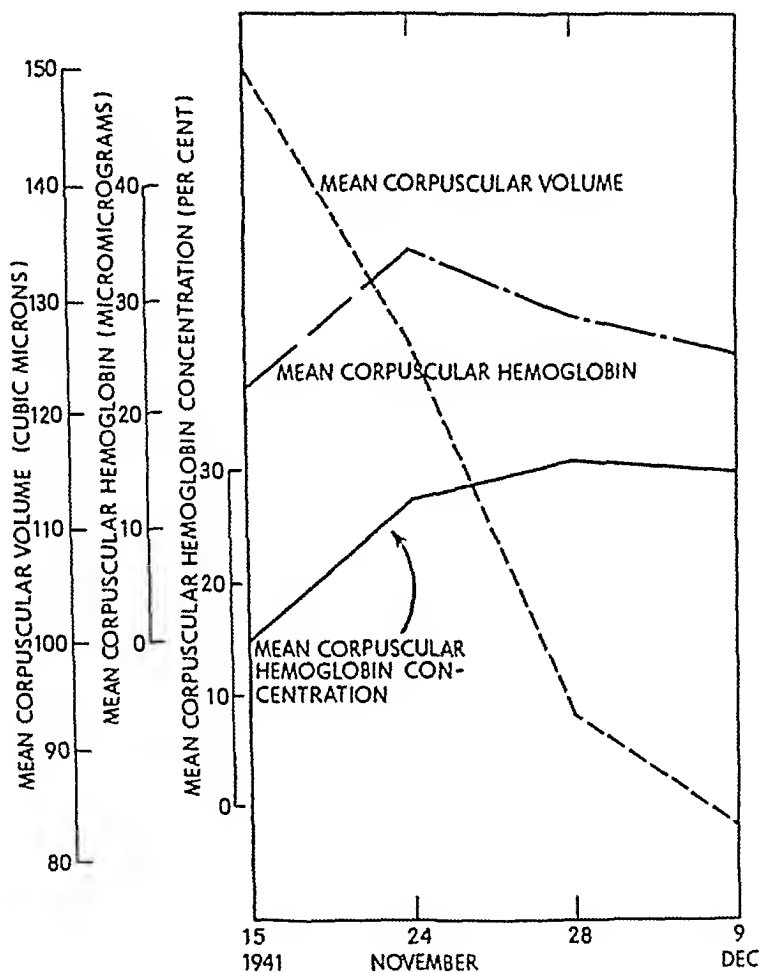


FIG. 6

Case 2. Mean corpuscular values in recovery phase of *Bartonella bacilliformis* anemia, demonstrating the remarkable change from macrocytosis to normal values.

A massive fatal invasion of *Bartonella bacilliformis* is observed in this child who died on the tenth day of the disease with an anemia with nearly one-half million red cells almost all parasitized by bartonellae.

Thrombocytopenia occurred in eight cases with purpura and external hemorrhages.<sup>13</sup> These hemorrhages were transitory and lasted no more than two weeks, being parallel to the drop of the platelet count. Fatal Cases 4 and 5 which follow illustrate thrombocytopenic purpura occurring in *Bartonella bacilliformis* anemia.

## Case 4

A S., an 18 year old farm helper, referred his disease to a traumatic accident on the dorsal surface of the right foot, which bled constantly. A local physician diagnosed gangrene of the foot. Careful interrogation, however, disclosed that this patient had been living for three months in an area where Carrion's disease is endemic and three days before receiving the foot wound, he had experienced general malaise and fever. He continued to work despite this symptomatology plus polydipsia and anorexia. After the accident, he noticed purpuric spots in the skin, epistaxis, bleeding of the gums, and a bloody discharge through the wound of the foot. On March 16, physical examination disclosed a well developed and well nourished, slightly jaundiced male, with many purpuric spots in the skin, bleeding of the gums, marked anemia, and petechial hemorrhage in the conjunctiva. Lymph nodes, of 5 to 15 mm, were palpable in the inguocrural, axillary, epitrochlear and cervical regions. The blood pressure was normal, the pulse 130 and the respirations 42 per minute. Over the first interosseal space on the right foot, there was a contusive wound 2 cm long and 1 cm deep with irregular borders, surrounded by an ecchymotic area of about 4 cm in diameter without signs of inflammation. Many other nontraumatic ecchymotic areas were spread over the body, especially on the anterior wall of the thorax. Coagulation time was thirteen minutes and bleeding time twenty-four minutes. Cultures for other bacteria besides *Bartonella bacilliformis* were negative. The peripheral blood had 0.97 red blood cells, 3,500 white blood cells and the differential was 56 neutrophils, 2 monocytes and 42 lymphocytes. There were 2 orthochromatic normoblasts and 1 plasma cell per 100 white blood cells. There was marked anisocytosis, poikilocytosis and anisochromia (table 1A). There were 50,000 platelets. No bartonellae were found in the red cells, nevertheless, Carrion's disease was diagnosed. The patient was given coagulants, fluids and anti-tetanic sera. He died on the second day of his admission. The gross pathologic diagnoses were terminal pulmonary edema, multiple petechial and ecchymotic hemorrhages, ecchymotic wound of the right foot and Carrion's disease. The stained smears of the spleen had many *Bartonella bacilliformis*.

This case illustrates a fatal thrombocytopenic purpura during a severe *Bartonella bacilliformis* anemia.

## Case 5

M. H., a 17 year old farm worker, after spending a month in a locality where Carrion's disease is endemic, on April 15, 1939, noticed fever, malaise and profuse sweating, symptoms which continued on the following days. The patient became anemic, slightly jaundiced and was brought into the hospital in a very critical condition on April 10, with convulsions, marked mental confusion, followed by a loss of consciousness. He appeared to be a well-nourished, well developed, seriously ill boy with a very marked anemia. There were many tiny petechial hemorrhages in the conjunctiva, pulsation of the arteries of the neck, functional murmurs over the precordium, and a slight yellow discoloration of the skin and sclerae. The pulse was 104 per minute, the blood pressure 120 over 70, the respirations were shallow, regular and 23 per minute. Examination of the spinal fluid on admission showed albumin 0.09 Gm per cent, glucose 0.44 per cent, urea 0.25 per cent, chlorides 0.4 mg per cent and 1 lymphocyte per cu mm. The Pandy reaction was negative. Typhoid and paratyphoid agglutinations were negative. The red blood count was 0.765 millions/cu mm (table 1A).

The patient continued with insomnia, a very annoying pulsating feeling in the head and ears, cramps in the epigastrium and right upper quadrant of the abdomen. The anemia increased and the general condition of the patient failed. The slight subicteric color of the sclera and skin disappeared and the icteric index became normal. No bartonellae were found in the red blood cells. The lymph nodes of the neck, inguinal and epitrochlear regions became enlarged. The fever oscillated between 36 and 40°C. On April 18, vomiting and headaches appeared with cough, rhonchi and crepitation of the lungs and on the April 22 many pinpoint purpuric spots were seen in the skin and conjunctiva. The patient became dyspneic and died on May 23. Autopsy showed the following prominent features: hydropericardium of nearly 1.5 liters, extensive petechial hemorrhages in many organs, such as the lungs, liver and spleen, external and internal hydrocephalus. *Ascarides lumbricoides* were found in the intestine.

This case illustrates a fatal recurrence of anemia due to thrombocytopenic purpura while the bartonellae were disappearing from the erythrocytes.

## "CRITICAL STAGE"

The term "critical stage" of bartonella bacilliformis anemia has been applied to the period of transition in which the organism suddenly disappears from the red cells.<sup>29-36</sup> The mechanism of this change is controversial, but it is a fact that within a few days the bartonellae may disappear from the peripheral erythrocytes. The hematologic signs of this transition are as follows: (1) A change in the form of the bartonellae from a bacilliform to a coccoid form (originally described by Barton<sup>3</sup> in a report published from Lima in 1908) occurs with the appearance of sphere, hour glass, pear shape and granule forms. These are the coccoid bartonella. (2) Decrease in the number of the parasitized erythrocytes and in the number of bartonellae on each erythrocyte. (3) An increase in the erythrocyte count. (4) A reduction in the indirect hyperbilirubinemia to normal. (5) An increase in the number of reticulocytes. (6) A decrease in the macrocytosis, later in the disease the erythrocytes regain normal size and even have a tendency to microcytosis. (7) A lymphocytosis, the monocytes and eosinophiles reappearing. (8) A shift of the polymorphonuclear series to the "right," a characteristic which persists during the rest of the disease.

Clinically, corresponding with this transition, the fever disappears, the subicteric tinge of the skin and sclera disappear leaving an intense earthen gray pallor. With the prompt rise in the number of erythrocytes the symptoms of anemia, such as fainting, dizziness, tinnitus, etc., disappear, as well as the hemic heart murmurs, the blood pressure rises. The patient appears to be convalescent. However, this sequence of events does not always occur, for clinical improvement may not parallel the disappearance of the bartonellae from the erythrocytes. There may be an increased severity of the clinical course of the disease, which is due to intercurrent infections, or to an atypical course of the disease itself. Occasionally, there is clinically an increased severity of the disease, coexisting with the favorable hematologic findings of the critical stage, despite the fact that there is no evidence of intercurrent infections. In these cases, one finds fever, vomiting, tachycardia, insomnia, delirium and psychomotor irritability. This occurred in Case 6, which terminated fatally.

## Case 6

J. M., an 18 year old female cook, lived for four months before becoming ill in an area where Carrion's disease is endemic. On October 6, 1939, she developed a headache followed by a chill, fever, malaise and profuse sweating. Despite the persistence of these symptoms, the patient did not stay in bed, the chills did not recur but the fever was continuous. She developed anemia and a slight yellow discoloration of the skin. On admission to the hospital on October 18, 1939, she appeared well nourished and well developed but was markedly confused mentally. She had severe anemia, slight jaundice, pronounced pulsations of the arteries of the neck and epigastrium, very dry skin, hemic murmurs over the precordium. The blood pressure was 115 over 70. The pulse was 120 per minute, the respirations were regular and superficial, 20 per minute. On October 20, the red count was 1.8 millions, hemoglobin 7.8 grams per cent, the leukocytes 6,080 (table 1A). The red blood cells showed a few bacilliform and coccoid bartonellae. No lymph nodes were palpable. The patient's condition rapidly failed, there was continuous vomiting and diarrhea, rigidity of the muscles of the neck, insomnia, motor excitability and headaches. She developed a deep coma progressively, nystagmus with marked increase in muscular tonicity of the neck and extremities. She had negative tendon reflexes and no Babinski response. A Cheyne-Stokes respiration

developed. The blood cultures were negative on several occasions for bacteria other than *Bartonella bacilliformis*. The detailed information of findings at the autopsy have been reported extensively elsewhere.<sup>5</sup> No definite complications could be found.

An unusual fatal, short failing, hyperthermic course of the disease is illustrated in this case without apparent intercurrent infection. At the time the bartonellae were disappearing from the erythrocytes, the patient developed symptoms frequently found in cases with intercurrent salmonella infection.

It is not known whether the bartonella infection itself is responsible for death in these cases. The interaction between the bartonellae and the reticulo-endothelial system has been studied extensively.<sup>40-45</sup> The bartonellae are found in large num-

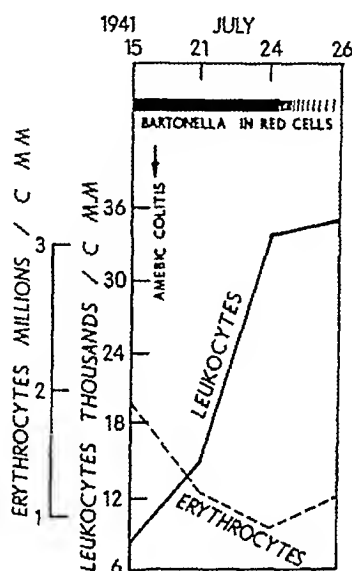


FIG 7

Leukocytosis occurring before death in case with *Bartonella bacilliformis* anemia complicated by amebic colitis.

At the top, in the bar representing *Bartonella* in red cells, solid black indicates heavy concentration of *Bartonella*, gradually widening spaces indicate progressive diminishing number of *Bartonella*.

bers in the phagocytic cells, such as the Kupffer cells of the liver, littoral cells of the spleen and of the lymph nodes, indicating clearly that the activity of the bartonellae is centered in the reticulo-endothelial system (figs 8-12).

Intercurrent infections may develop in these patients during the critical period of the anemia when there appears to be a lowered resistance of the host to invading organisms harbored in the gastrointestinal tract. These organisms frequently produce a fatal septicemia. In these cases the symptoms are bizarre and confusing. There is usually a rise in the temperature, tachycardia, diarrhea, dysentery, psychomotor excitability, followed shortly by death. There is a sharp leukocytosis with shift to the left in the polymorphonuclear series and a fall in the number of reticulocytes and normoblasts. This is well illustrated in figure 7, complicated by amebic colitis. Blood cultures are very helpful in determining the complicating organism. In this series of 30 cases, intercurrent infections were detected in 15, of whom 3 developed malaria which was promptly controlled by quinine therapy,

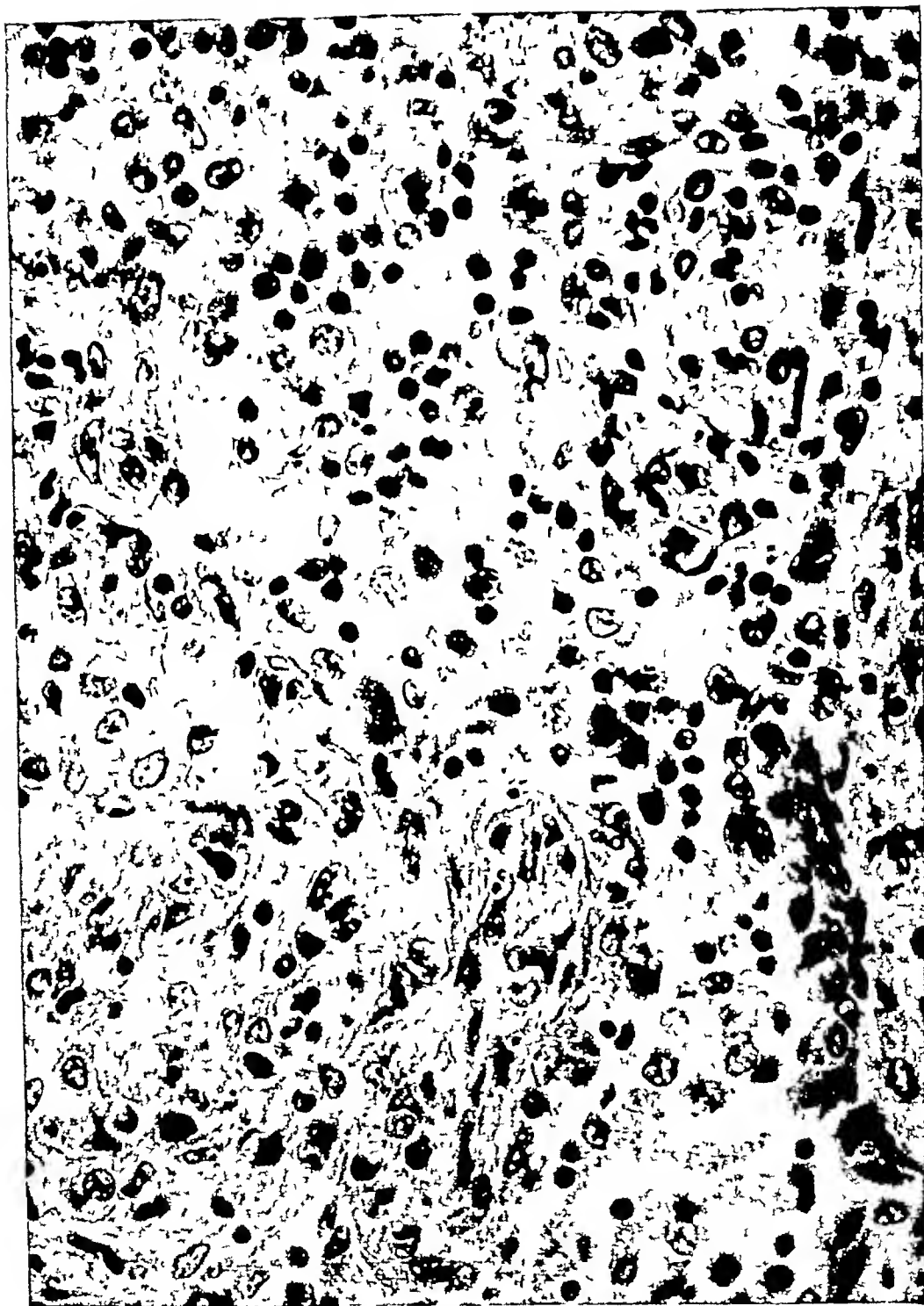


FIG 8

Large splenic blood sinus containing many nucleated red blood cells. The large dark littoral cells contain *Bartonella bacilliformis*. Giemsa stain  $\times 580$ .

2 out of 3 died with typhoid fever, 2 died of a *Salmonella schottmulleri* infection, 3 died with amebic colitis, and in 3 cases the necropsy examination suggested an anaerobic septicemia due to an undetermined organism.





FIG 9

Colony-like masses, each composed of innumerable *Bartonella bacilliformis*, in littoral cells lining a small splenic sinus Giemsa stain  $\times 1275$

In 9 fatal cases, no bacterial or parasitic intercurrent infections were demonstrated. Peripheral collapse occurred in 1 patient who died, and thrombocyto-

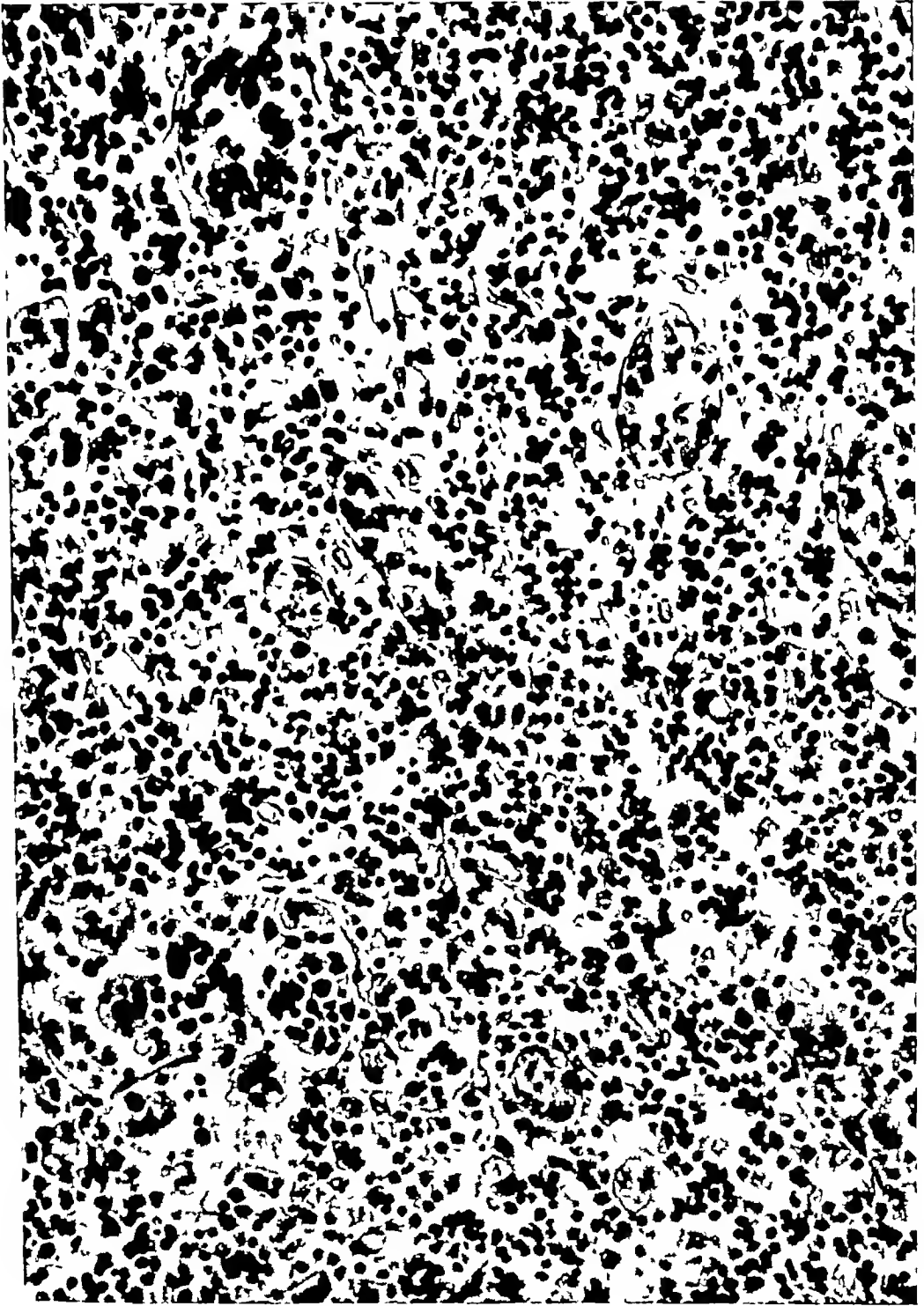


FIG 10

Bartonella-laden littoral cells lining small blood vessels in a lymph node. Giemsa stain  $\times 335$

penic purpura was the cause of death in two. In 6 others, the cause of death was unknown.

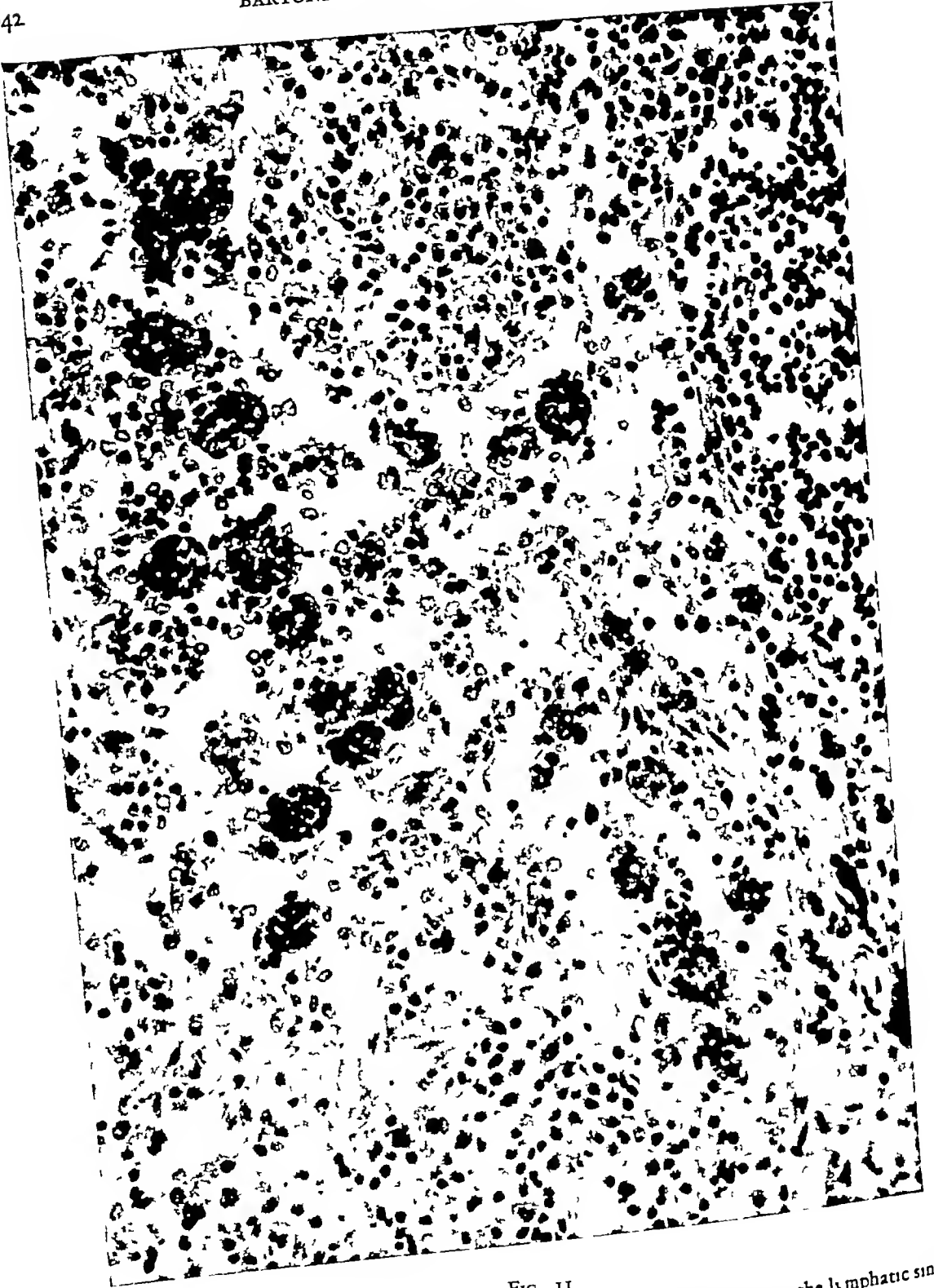


FIG 11

Section of a lymph node showing large macrophages filled with erythrocytes in the lymphatic sinuses  
 Bartonellae are seen in the littoral cells of the lymphatic sinuses Jimenez stain  $\times 325$

#### PATHOGENESIS

*Bartonella bacilliformis* anemia is a hemolytic anemia in which the destruction of erythrocytes is dependent on the presence of *bartonella bacilliformis* on the

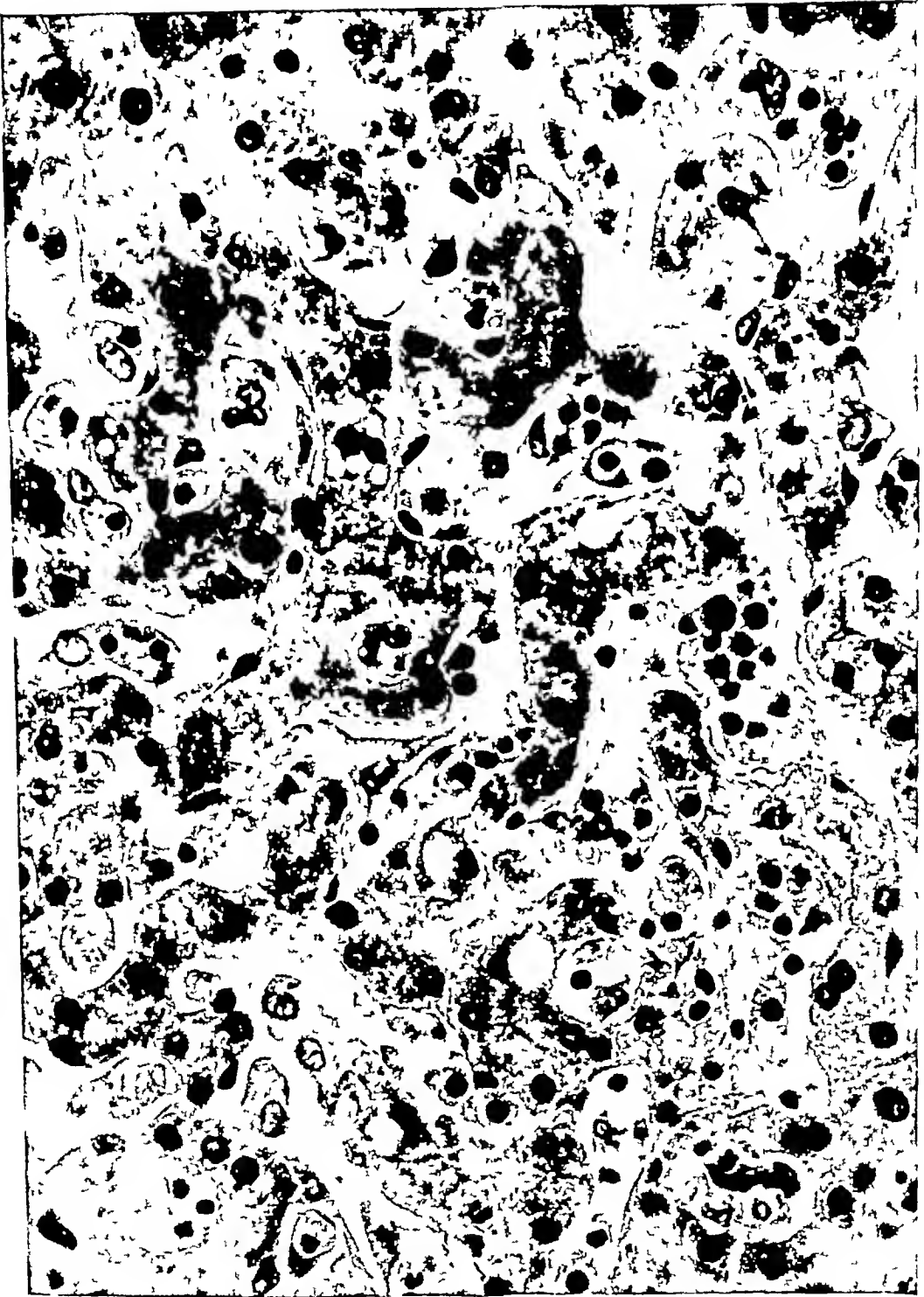


FIG. 12.

Section of the liver showing large Kupffer cells, which contain erythrocytes, nucleated red blood cells, and small aggregates of *Bartonella bacilliformis* Giemsa stain  $\times 570$

erythrocytes. The rate of destruction of the erythrocytes and the increased bilirubinemia<sup>15</sup> are directly proportional to the number of parasitized erythrocytes.<sup>15</sup> The anemia is macrocytic in type with signs of increased blood formation— young

granulocytes, polychromatophilia, reticulocytosis and nucleated red cells. Hypochromia is a frequent finding but did not occur in several cases of this series (table 1). There is increased urobilinogen excretion in the feces. Hurtado et al.<sup>15</sup> have shown that the fragility of the erythrocytes is normal in this disease. There are no spherocytes and enlargement of the spleen clinically does not occur in the cases without intercurrent infections.<sup>7, 13</sup> Hemoglobinuria does not occur. An early suggestion was that the bartonella bacilliformis anemia was "Addisonian" anemia. Guzman-Barron<sup>16</sup> demonstrated the presence of Castle's principle in the gastric secretion, and careful hematologic studies<sup>15</sup> have shown that the anemia is normoblastic and not megaloblastic and is accompanied by marked reticulocytosis. Histologically there is evidence of erythrocyte destruction in the reticulo-endothelial system as the Kupffer cells, littoral cells of the spleen and lymph nodes and other phagocytes appear filled with bartonellae and erythrocytes (figs 8-12).

#### PROGNOSIS

*Bartonella bacilliformis* anemia has a severe prognosis, due mostly to the frequency of intercurrent infection produced by varied organisms from the gastrointestinal tract. The host's defenses, already lowered by the bartonellosis, are overwhelmed by the subsequent infection.

In 30 cases studied in this series, 22 died, a mortality of 73 per cent. Fifteen of these cases had intercurrent infection, and of these, 11 died with septicemia of gastrointestinal origin. In a group of 15 cases without evidence of any concurrent infection, 9 died. In only 2 cases (Cases 3 and 5) could the death be explained by the virulence of the infection and the intensity of the anemia. Case 5 apparently died of a thrombocytopenic purpura with 440,000 erythrocytes. In the remaining 7 fatal cases the intensity of the anemia was not severe enough to explain the fatal course, the severity of the disease apparently being due to the bartonella infection itself.

#### TREATMENT

At present, there is no specific therapeutic agent which acts against the bartonella bacilliformis and the treatment used is largely symptomatic. A great deal of therapeutic effort has been directed toward stimulating the bone marrow. This is of dubious importance in cases without any complication, as the main problem in bartonella bacilliformis anemia is not the formation of red cells but the destruction of erythrocytes. The reticulocytosis is sufficient evidence of regeneration. Case 1 in this series without specific treatment or marrow stimulation showed an extraordinary degree of erythrocytic regeneration, this is seen in every case without intercurrent infection. The sudden transition occurring naturally in the course of the disease with disappearance of the bartonellae from the red cells may give the fallacious impression that a given drug or a treatment is responsible for such change. There is some evidence that liver extracts<sup>15</sup> may accelerate the erythrocyte regeneration.

Blood transfusions appear to be indicated, however, up to the present this method of treatment has been used only occasionally. This is largely due to the

prevailing theory<sup>35</sup> that the crisis of the anemia with the disappearance of the bartonella bacilliformis from the red cells is due to the immunity of the red cells toward the bartonellae. Blood transfusion, according to this theory, will alter the immunity since the donor cells are supposedly more susceptible and so more apt to be parasitized. This is not acceptable, as the erythrocytes have never been known to have immunologic activity. The immunologic changes which occur in the host in the critical stage produce the changes both in the shape and in the clearance of the bartonellae from the erythrocytes. This is supported by the fact that the anemia does not recur in the course of the disease, even if fatal intercurrent infection occurs.<sup>14</sup> Small blood transfusions of 300 to 400 cc. used in a very few cases with severe anemia and thrombocytopenia in my experience have proved to be a life-saving procedure. Unfortunately, larger amounts of blood were not available to test any of the patients included in this series. The other main objective in the treatment of bartonella bacilliformis anemia should be the prevention of intercurrent infection. Penicillin has lately been reported in a small number of cases to produce good results in the treatment of the disease.<sup>47-50</sup> Further studies are necessary to evaluate the action of this drug. In the future, studies should be directed toward the control of the bartonella bacilliformis, as well as the prevention of intercurrent infection.

#### DISCUSSION

A severe hemolytic anemia may occur during the invasive stage of Carrion's disease. The pathogenic organism, bartonella bacilliformis, is found in the erythrocytes and on the cells of the reticulo-endothelial system. It has been previously stated that bartonella bacilliformis anemia occurs at any stage of Carrion's disease.<sup>2, 31 et al</sup> However, there are no proven cases in which this anemia has occurred except in the invasive stage of the disease. Anemias which occur later in the pre-eruptive and eruptive stages, were found in a previous study<sup>13</sup> to have a different etiology and different morphologic and clinical characteristics. The anemias without bartonellas in the erythrocytes are due to intercurrent infections, hemorrhages, thrombocytopenic purpura or other causes. Recurrences of bartonella anemia did not occur in these series of patients even in cases with fatal intercurrent infections. This is another important fact against the concept<sup>36</sup> that blood transfusions should not be given to these patients because of the danger of reinvasion of the erythrocyte by bartonellae. On the contrary it seems from this study that these patients will be greatly benefited by repeated blood transfusions from 600 to 1000 cc. as a supportive therapy against the bartonella infection and the anemia. With the disappearance of bartonellae from the erythrocytes, which occurs during the natural course of the bartonella infection, the red cell hemolysis ceases and there is a spontaneous prompt recovery.

The transition period in which the bartonellae suddenly disappear from the erythrocytes in the circulating blood is characterized by a very low resistance of the host to secondary infection. This accounts for a considerable proportion of mortality in this disease, as is illustrated by 11 deaths occurring in 12 septicemic cases. In only 2 cases (Cases 3 and 5) in which the erythrocyte counts were ap-

proximately 5 million/cu mm could death be attributed to the severity of the anemia. In all the other cases the severity of the disease was due to the bartonella infection or to intercurrent infection and could not be related to the intensity of the anemia.

### SUMMARY

*Bartonella bacilliformis* anemia (Oroya fever) is a febrile hemolytic anemia with distinguishing clinical and hematologic characteristics. It occurs as an infrequent clinical form during the invasive stage. The onset is variable with or without chills, followed by a moderate temperature which does not parallel the intensity of the anemia. Hemorrhages, petechial spots, epistaxis may occur and are due to thrombocytopenia. Clouding of the sensorium and delirium are rather uncommon. There is a generalized lymphadenopathy but no splenomegaly.

The anemia is macrocytic and frequently hypochromic with signs of intense blood formation: young granulocytes, polychromatophilia, reticulocytosis and nucleated red cells. The reticulocytosis may increase to 50 per cent. The pathognomonic sign of the disease is the presence of *bartonella bacilliformis* on the erythrocytes. The leukocyte count varies, slight leukocytosis is not uncommon but marked leukocytosis is extremely rare in cases without intercurrent infections. There is a shift of the polymorphonuclear series to the left, characterized by the presence of myeloblasts, myelocytes and metamyelocytes. The anemia is normoblastic and not megaloblastic. It is hemolytic and the destruction of erythrocytes is dependent on the presence of *bartonella bacilliformis* on the erythrocytes. There is no spherocytosis and the fragility of the erythrocytes is normal. Histologically there is evidence of erythrocyte phagocytosis in the cells of the reticulo-endothelial system, the Kupffer cells, the littoral cells of the spleen and the lymph nodes.

The disappearance of the bartonellae from the erythrocytes occurs in a very few days and is called "critical stage" of the anemia. The hematologic changes of this transition are as follows: a change in the shape of the bartonellae from the 'bacilliform' to 'coccoid' form before complete clearance will take place, an increase in the erythrocyte count, reduction in the indirect hyperbilirubinemia to normal, increase in the number of reticulocytes, reversion to normocytosis, lymphocytosis, reappearance of monocytes and eosinophiles, a 'shift to the right' of the polymorphonuclear series. Corresponding with the clearance of bartonella from the erythrocytes, the symptoms dependent on the anemia as well as the fever disappear. Clinical improvement, however, may not parallel the clearance of bartonellae because of intercurrent infections or an atypical course of the bartonellosis itself.

*Bartonella bacilliformis* anemia has a very severe prognosis, due largely to the occurrence of intercurrent infection by enteric organisms. The treatment today is largely symptomatic as there is no specific agent against the bartonellae bacilliformis infection.

It is suggested that the prognosis may be improved by the use of adequate blood transfusions and the prophylactic use of antibiotics to control intercurrent in

fection At present there is no specific agent against bartonella bacilliformis anemia

### CONCLUSIONS

1 Bartonella bacilliformis anemia (Oroya fever) is a type of hemolytic anemia in which the pathogenic organism of the disease is found in the circulating erythrocytes and in the cells of the reticulo-endothelial system

2 Bartonella bacilliformis anemia is macrocytic, frequently hypochromic and occurs only during the invasive stage of Carrion's disease and does not recur in the course of the disease Other anemias which develop later have different clinical and hematologic characteristics

3 The transition period in which the bartonellae suddenly disappear from the erythrocytes depends on immunologic changes of the host toward the bartonella infection

4 The prognosis of bartonella anemia is very grave, as is indicated by a mortality rate of 73 per cent in this series of 30 cases This very severe prognosis is largely due to intercurrent infection which occurred in 50 per cent of the cases studied The most severe prognosis was found in cases with septicemias from enteric organisms, as indicated by 11 deaths out of 12 cases

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slightly icteric. There were abnormally intense carotid arterial pulsations synchronous with the pulse, and functional hemic murmurs over all areas of the heart, the blood pressure was 110/50, the pulse 120 per minute, and the respirations were accelerated and very shallow. The patient rapidly became confused, had psychomotor excitability, the anemia increased and the fever continued between 37 and 38.2 C. He complained of occipital headache, precordial oppression and insomnia, epistaxis occurred on several occasions. On July 21, 1938, the erythrocyte count totaled only 0.575 million per cu mm, the hemoglobin was 3.3 grams per cent and the leukocytes 7,600. There was a macrocytosis of 198.1 cubic microns and 461 normoblasts per 100 leukocytes. The red cells showed marked anisocytosis, poikilocytosis and polichromasia with many bartonella bacilliformis. The Wassermann and Kahn reactions were strongly positive. The symptoms continued unchanged until July 28. On that day, no bartonellae

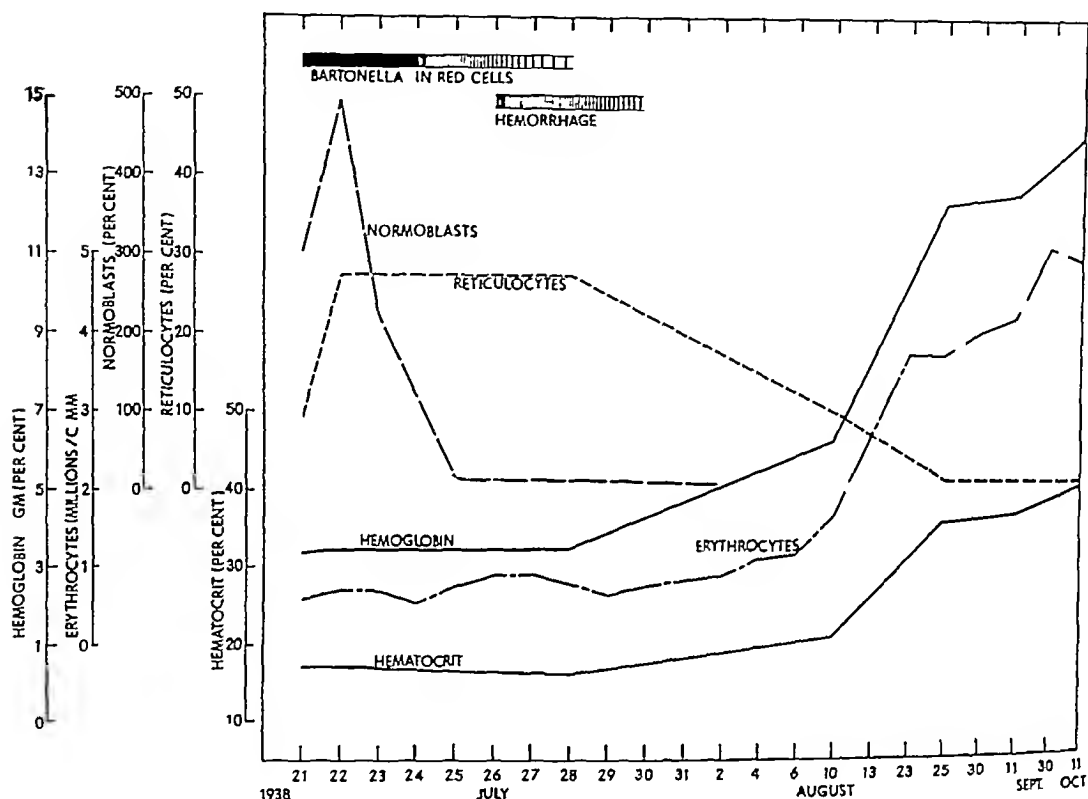


FIG 3

Case 1. Critical and recovery phases of *Bartonella bacilliformis* anemia, showing the marked increase of the red cells, hemoglobin and hematocrit after the disappearance of the *Bartonella bacilliformis*.

At the top, in the bars representing *Bartonella* in red cells, and hemorrhage, solid black indicates heavy concentration of *Bartonella bacilliformis*, gradually widening spaces indicate progressive diminishing number of *Bartonella bacilliformis*.

were present in the erythrocytes. On July 29, the general condition of the patient became worse with insomnia and delirium during the night, he had severe epistaxis with loss of approximately 500 cc of blood, the platelets numbered 80,000, the red cells decreased from 0.82 to 0.62 million. The patient remained apathetic in bed and slight edema of the eyelids was noticeable for the first time. The temperature rose to 39 C. Blood cultures were negative for bacteria other than *Bartonella bacilliformis*. During the first week of August, his general condition improved strikingly despite the fever. The number of red cells increased considerably without any therapy, the symptoms faded and the patient developed a ravenous appetite. On October 11, 1938, before leaving the hospital, the erythrocyte count was 4.84 million/cu mm, the hemoglobin 13.8 grams per cent, the hematocrit 39.1 per cent. The mean corpuscular volume was 81, the leukocytes 5,240. Wassermann and Kahn reactions found strongly positive on admission were considered false. These tests later were repeatedly negative.

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This case illustrates a severe bartonella anemia with nearly a half million erythrocytes/cu mm, an unusual intensive erythrocytic regeneration and an exceptional macrocytosis. Nasal bleeding coexisted with thrombocytopenia. Despite a prompt recovery an unexplained febrile episode followed the disappearance of bartonellae from the erythrocytes.

Case 2 (Figs 5 and 6)

V. C. S., a 21 year old Peruvian male, on April 19, 1941, was sent for ten days to an area where Carion's disease and malaria are endemic. On July 18, he developed malaria which disappeared after three days of intense antimalarial treatment.

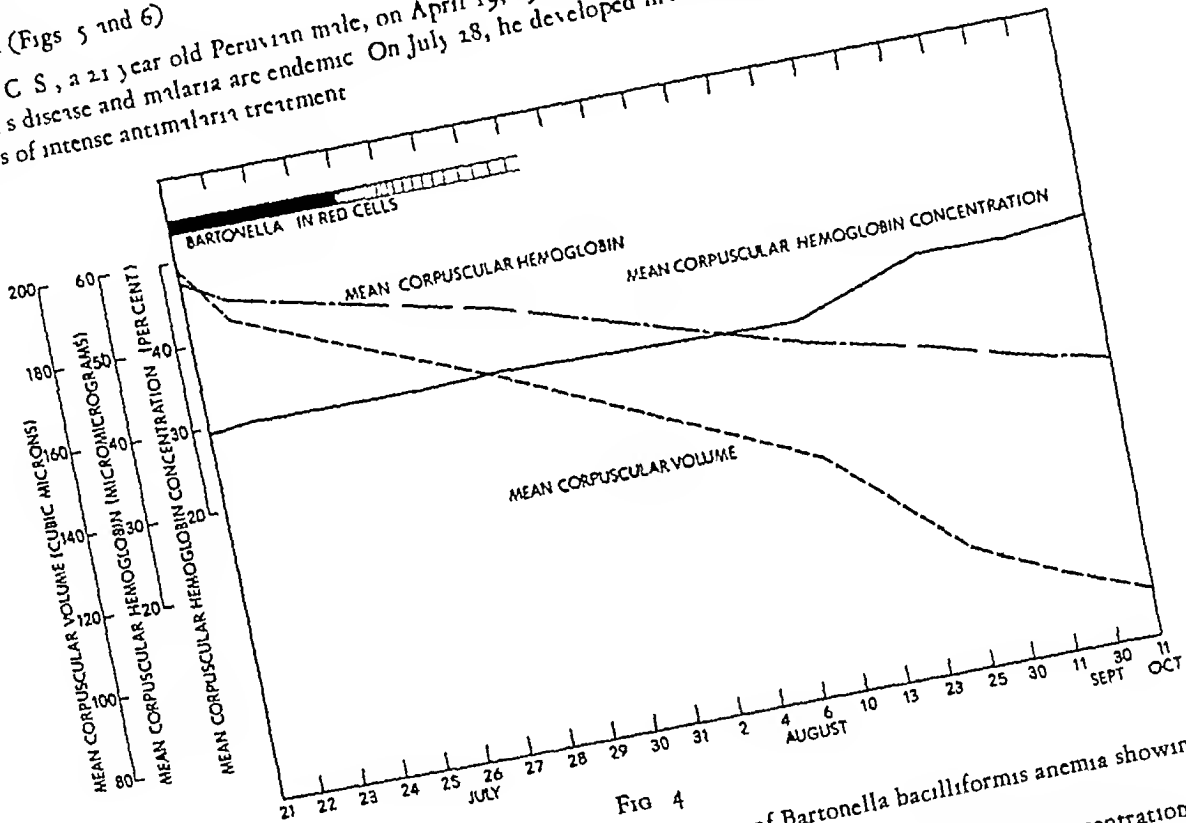


FIG 4

Case 1: Mean corpuscular values during the recovery phase of Bartonella bacilliformis anemia showing striking fall in the mean corpuscular volume. At the top, in the bar representing Bartonella in red cells, solid black indicates heavy concentration of Bartonella bacilliformis, gradually widening spaces indicate progressive diminishment of Bartonella bacilliformis.

On November 1, he again developed fever and malaise, preceded by a single chill. Mental stupor, marked psychomotor excitability and delirium soon developed, the fever being slight and continuous. On admission (November 8, 1941), the patient was mentally confused and in such a serious condition that it was thought he would die shortly. There was a very severe anemia and jaundice, functional murmurs were present all over the precordium, the blood pressure was within normal limits, the pulse was 120 and the respirations were regular, 16 per minute. Only a few lymph nodes less than 1 cm in diameter were palpated in both axillae. The urine was normal. The van den Bergh reaction for bilirubin was of an indirect type, and the Wassermann and Kahn reactions were negative. The red count was 0.785 millions/cu mm with 46 per cent reticulocytes, hemoglobin 17.5 grams (Evelyn photocolormeter), hematocrit 11.7 per cent, the mean corpuscular volume was 149.9 cubic microns, and icteric index 15. The white blood count was 13,060, with 58 polymorphonuclear neutrophils (2 myelocytes, 5 metamyelocytes, 15 filamented and 36 segmented cells) and 42 lymphocytes. There were 47 normoblasts per 100 leukocytes. Pronounced anisocytosis, poikilocytosis and polichromasia were found. A very small

percentage of erythrocytes contained coccoid bartonellae. After a few days, the existing bartonellae disappeared, the number of erythrocytes increased rapidly, the mean corpuscular values became normal, the slight jaundice cleared, the mental symptoms improved and the fever disappeared. In the last two weeks of December and the first week of January, he had transitory pains in the epiphysis of the long bones, in the short bones of the hands and feet and in the knees. He had paresthesias, the sensation of small worms in the skin. Two red spots not larger than a pinpoint were noticed on the skin, corresponding probably to verrugae in formation. No anemia was recorded the day the patient left the hospital, January 22, 1942.

This case illustrates a very prompt recovery after a severe bartonella bacilliformis anemia.

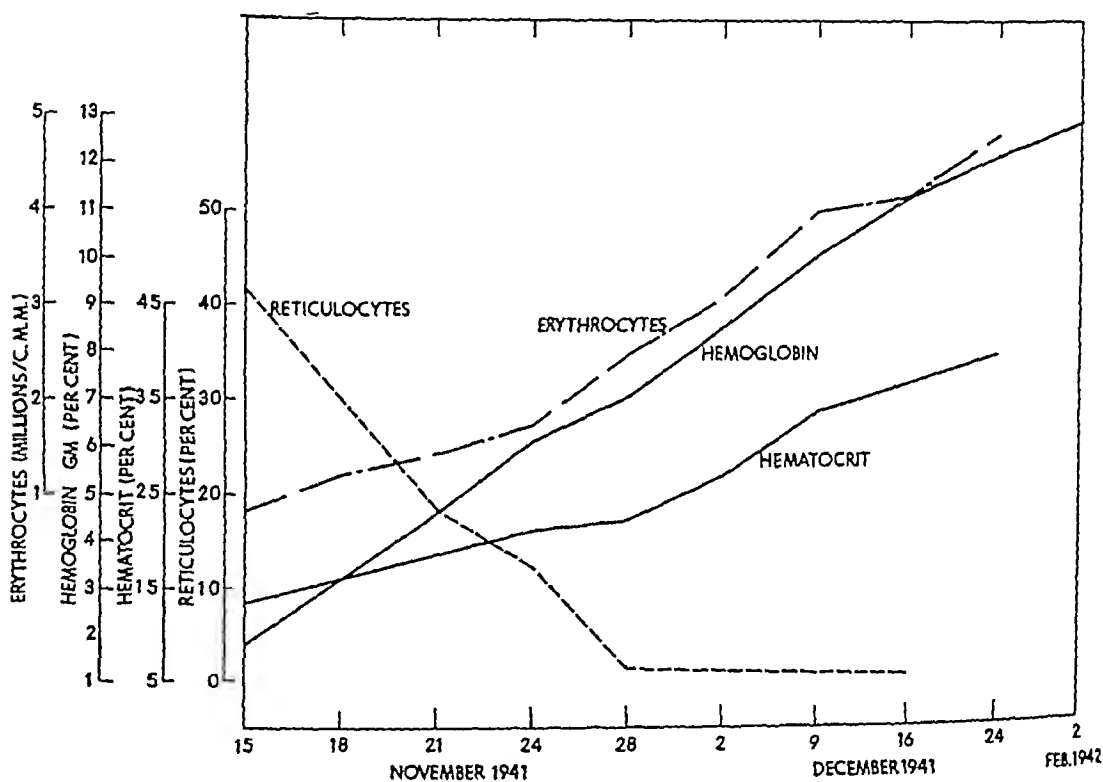


FIG 5

### Case 2. Recovery phase of Bartonella bacilliformis anemia

#### Case 3

R. H., a 7 year old native Peruvian boy, came from a town where Carrion's disease is endemic. On May 28, 1941, he developed malaise and lack of appetite, however, he continued going to school until June 1. His mother noticed that he had fever, had vomited several times and had become anemic and slightly jaundiced. Two days before admission he became restless and delirious. In view of the seriousness of this condition, he was brought to the Children's Hospital in Lima on June 8, 1941. Physical examination showed a very intense anemia. The sclerae of the eyes were slightly icteric, the pupils were dilated, but reacted well to light. He had meningeal signs, rigidity of the neck, maxillary trismus, loss of tendonous reflexes, and bilaterally absent Babinski reflexes. The lymph nodes in the neck were slightly enlarged, the liver was palpated 3.5 cm. below the costal margin in the right mid-clavicular line, there were marked carotid pulsations in the neck, together with functional murmurs all over the precordium. The urine was normal. On July 8, 1941, the erythrocyte count was 0.625 million, the leukocytes 35,000,

reticulocytes 20.2 per cent, hemoglobin 2.4 grams per cent ( Evelyn photocolormeter ), hematocrit 9.81, icterus index 20, mean corpuscular volume 158.55 cubic microns, mean corpuscular hemoglobin 38.40 micromicrograms, mean corpuscular hemoglobin conc. 24.22 per cent. The differential count was polymorphonuclear neutrophils 44 (5 myelocytes, 5 metamyelocytes, 25 filamented forms and 11 segmented cells), lymphocytes 54 per cent, promyelocytes 2. There were 1 erythroblast and 64 normoblasts per cent. There were pronounced anisocytosis, poikilocytosis and polichromasia. Ninety-six per cent of erythrocytes contained bartonella bacilliformis and as many as 19 bartonellae were present in each red cell (fig. 1). The patient died on July 8, 1941, and no autopsy could be obtained.

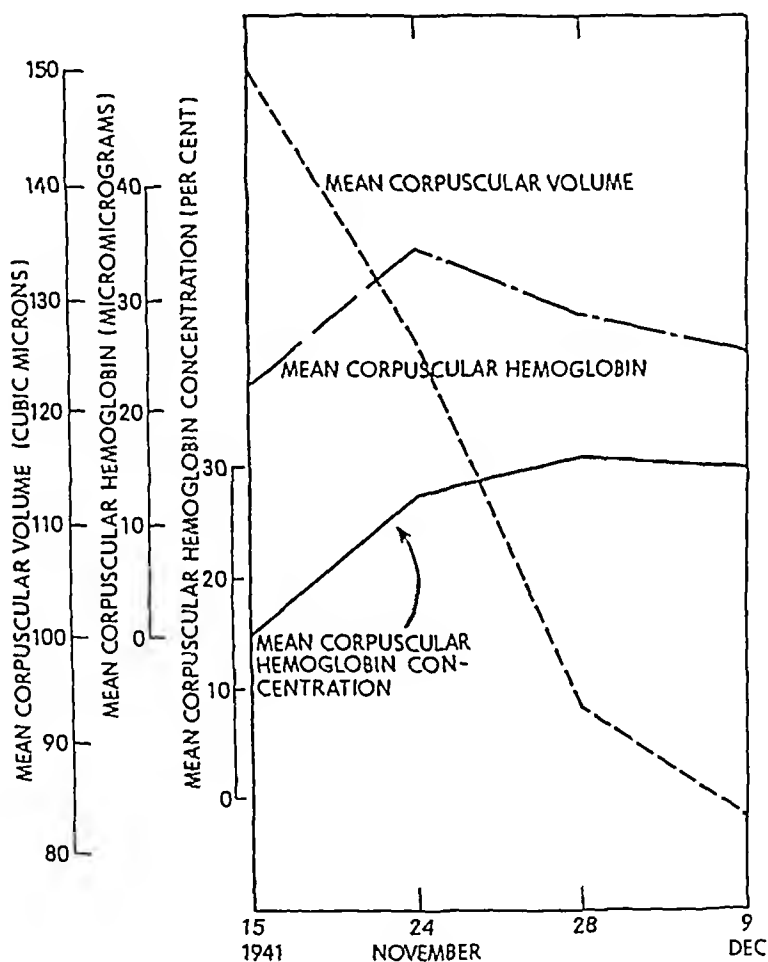


FIG 6

Case 2. Mean corpuscular values in recovery phase of *Bartonella bacilliformis* anemia, demonstrating the remarkable change from macrocytosis to normal values.

A massive fatal invasion of *bartonella bacilliformis* is observed in this child who died on the tenth day of the disease with an anemia with nearly one-half million red cells almost all parasitized by bartonellae.

Thrombocytopenia occurred in eight cases with purpura and external hemorrhages.<sup>13</sup> These hemorrhages were transitory and lasted no more than two weeks, being parallel to the drop of the platelet count. Fatal Cases 4 and 5 which follow illustrate thrombocytopenic purpura occurring in *bartonella bacilliformis* anemia.

## Case 4

A S, an 18 year old farm helper, referred his disease to a traumatic accident on the dorsal surface of the right foot, which bled constantly. A local physician diagnosed gangrene of the foot. Careful interrogation, however, disclosed that this patient had been living for three months in an area where Carrion's disease is endemic and three days before receiving the foot wound, he had experienced general malaise and fever. He continued to work despite this symptomatology plus polydipsia and anorexia. After the accident, he noticed purpuric spots in the skin, epistaxis, bleeding of the gums, and a bloody discharge through the wound of the foot. On March 16, physical examination disclosed a well developed and well nourished, slightly jaundiced male, with many purpuric spots in the skin, bleeding of the gums, marked anemia, and petechial hemorrhage in the conjunctiva. Lymph nodes, of 5 to 15 mm, were palpable in the inguino-cervical, axillary, epitrochlear and cervical regions. The blood pressure was normal, the pulse 130 and the respirations 42 per minute. Over the first interosseal space on the right foot, there was a contusive wound 2 cm long and 1 cm deep with irregular borders, surrounded by an ecchymotic area of about 4 cm in diameter without signs of inflammation. Many other nontraumatic ecchymotic areas were spread over the body, especially on the anterior wall of the thorax. Coagulation time was thirteen minutes and bleeding time twenty-four minutes. Cultures for other bacteria besides *Bartonella bacilliformis* were negative. The peripheral blood had 0.97 red blood cells, 3,500 white blood cells and the differential was 56 neutrophils, 2 monocytes and 42 lymphocytes. There were 2 orthochromatic normoblasts and 1 plasma cell per 100 white blood cells. There was marked anisocytosis, poikilocytosis and anisochromia (table 1A). There were 50,000 platelets. No bartonellae were found in the red cells, nevertheless, Carrion's disease was diagnosed. The patient was given coagulants, fluids and anti-tetanic sera. He died on the second day of his admission. The gross pathologic diagnoses were terminal pulmonary edema, multiple petechial and ecchymotic hemorrhages, ecchymotic wound of the right foot and Carrion's disease. The stained smears of the spleen had many *Bartonella bacilliformis*.

This case illustrates a fatal thrombocytopenic purpura during a severe *Bartonella bacilliformis* anemia.

## Case 5

M H, a 17 year old farm worker, after spending a month in a locality where Carrion's disease is endemic, on April 15, 1939, noticed fever, malaise and profuse sweating, symptoms which continued on the following days. The patient became anemic, slightly jaundiced and was brought into the hospital in a very critical condition on April 10, with convulsions, marked mental confusion, followed by a loss of consciousness. He appeared to be a well-nourished, well developed, seriously ill boy with a very marked anemia. There were many tiny petechial hemorrhages in the conjunctiva, pulsation of the arteries of the neck, functional murmurs over the precordium, and a slight yellow discoloration of the skin and sclerae. The pulse was 104 per minute, the blood pressure 120 over 70, the respirations were shallow, regular and 23 per minute. Examination of the spinal fluid on admission showed albumin 0.09 Gm per cent, glucose 0.44 per cent, urea 0.25 per cent, chlorides 0.4 mg per cent and 1 lymphocyte per cu mm. The Pandy reaction was negative. Typhoid and paratyphoid agglutinations were negative. The red blood count was 0.765 millions/cu mm (table 1A).

The patient continued with insomnia, a very annoying pulsating feeling in the head and ears, cramps in the epigastrium and right upper quadrant of the abdomen. The anemia increased and the general condition of the patient failed. The slight subicteric color of the sclera and skin disappeared and the icteric index became normal. No bartonellae were found in the red blood cells. The lymph nodes of the neck, inguinal and epitrochlear regions became enlarged. The fever oscillated between 36 and 40 C. On April 18, vomiting and headaches appeared with cough, rhonchi and crepitation of the lungs and on the April 22 many pinpoint purpuric spots were seen in the skin and conjunctiva. The patient became dyspneic and died on May 23. Autopsy showed the following prominent features: hydropericardium of nearly 1.5 liters, extensive petechial hemorrhages in many organs, such as the lungs, liver and spleen, external and internal hydrocephalus. *Ascaris lumbricoides* were found in the intestine.

This case illustrates a fatal recurrence of anemia due to thrombocytopenic purpura while the bartonellae were disappearing from the erythrocytes.

## "CRITICAL STAGE"

The term "critical stage" of bartonella bacilliformis anemia has been applied to the period of transition in which the organism suddenly disappears from the red cells.<sup>29-35</sup> The mechanism of this change is controversial, but it is a fact that within a few days the bartonellae may disappear from the peripheral erythrocytes. The hematologic signs of this transition are as follows: (1) A change in the form of the bartonellae from a bacilliform to a coccoid form (originally described by Barton<sup>3</sup> in a report published from Lima in 1908) occurs with the appearance of sphere, hour glass, pear shape and granule forms. These are the coccoid bartonella. (2) Decrease in the number of the parasitized erythrocytes and in the number of bartonellae on each erythrocyte. (3) An increase in the erythrocyte count. (4) A reduction in the indirect hyperbilirubinemia to normal. (5) An increase in the number of reticulocytes. (6) A decrease in the macrocytosis, later in the disease the erythrocytes regain normal size and even have a tendency to microcytosis. (7) A lymphocytosis, the monocytes and eosinophiles reappearing. (8) A shift of the polymorphonuclear series to the "right," a characteristic which persists during the rest of the disease.

Clinically, corresponding with this transition, the fever disappears, the subicteric tinge of the skin and sclera disappear leaving an intense earthen gray pallor. With the prompt rise in the number of erythrocytes the symptoms of anemia, such as fainting, dizziness, tinnitus, etc., disappear, as well as the hemic heart murmurs, the blood pressure rises. The patient appears to be convalescent. However, this sequence of events does not always occur, for clinical improvement may not parallel the disappearance of the bartonellae from the erythrocytes. There may be an increased severity of the clinical course of the disease, which is due to intercurrent infections, or to an atypical course of the disease itself. Occasionally, there is clinically an increased severity of the disease, coexisting with the favorable hematologic findings of the critical stage, despite the fact that there is no evidence of intercurrent infections. In these cases, one finds fever, vomiting, tachycardia, insomnia, delirium and psychomotor irritability. This occurred in Case 6, which terminated fatally.

## Case 6

J. M., an 18 year old female cook, lived for four months before becoming ill in an area where Carrion's disease is endemic. On October 6, 1939, she developed a headache followed by a chill, fever, malaise and profuse sweating. Despite the persistence of these symptoms, the patient did not stay in bed, the chills did not recur but the fever was continuous. She developed anemia and a slight yellow discoloration of the skin. On admission to the hospital on October 18, 1939, she appeared well nourished and well developed but was markedly confused mentally. She had severe anemia, slight jaundice, pronounced pulsations of the arteries of the neck and epigastrium, very dry skin, hemic murmurs over the precordium. The blood pressure was 115 over 70. The pulse was 120 per minute, the respirations were regular and superficial, 20 per minute. On October 20, the red count was 1.8 millions, hemoglobin 7.8 grams per cent, the leukocytes 6,080 (table 1A). The red blood cells showed a few bacilliform and coccoid bartonellae. No lymph nodes were palpable. The patient's condition rapidly failed, there was continuous vomiting and diarrhea, rigidity of the muscles of the neck, insomnia, motor excitability and headaches. She developed a deep coma progressively, nystagmus with marked increase in muscular tonicity of the neck and extremities. She had negative tendon reflexes and no Babinski response. A Cheyne-Stokes respiration



developed. The blood cultures were negative on several occasions for bacteria other than *Bartonella bacilliformis*. The detailed information of findings at the autopsy have been reported extensively elsewhere.<sup>5</sup> No definite complications could be found.

An unusual fatal, short failing, hyperthermic course of the disease is illustrated in this case without apparent intercurrent infection. At the time the bartonellae were disappearing from the erythrocytes, the patient developed symptoms frequently found in cases with intercurrent salmonella infection.

It is not known whether the bartonella infection itself is responsible for death in these cases. The interaction between the bartonellae and the reticulo-endothelial system has been studied extensively.<sup>40-45</sup> The bartonellae are found in large num-

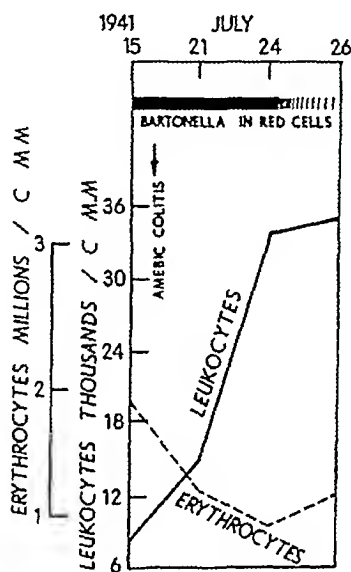


FIG 7

Leukocytosis occurring before death in case with *Bartonella bacilliformis* anemia complicated by amebic colitis.

At the top, in the bar representing *Bartonella* in red cells, solid black indicates heavy concentration of *Bartonella*, gradually widening spaces indicate progressive diminishing number of *Bartonella*.

bers in the phagocytic cells, such as the Kupffer cells of the liver, littoral cells of the spleen and of the lymph nodes, indicating clearly that the activity of the bartonellae is centered in the reticulo-endothelial system (figs 8-12).

Intercurrent infections may develop in these patients during the critical period of the anemia when there appears to be a lowered resistance of the host to invading organisms harbored in the gastrointestinal tract. These organisms frequently produce a fatal septicemia. In these cases the symptoms are bizarre and confusing. There is usually a rise in the temperature, tachycardia, diarrhea, dyspnea, psychomotor excitability, followed shortly by death. There is a sharp leukocytosis with shift to the left in the polymorphonuclear series and a fall in the number of reticulocytes and normoblasts. This is well illustrated in figure 7, complicated by amebic colitis. Blood cultures are very helpful in determining the complicating organism. In this series of 30 cases, intercurrent infections were detected in 15, of whom 3 developed malaria which was promptly controlled by quinine therapy.

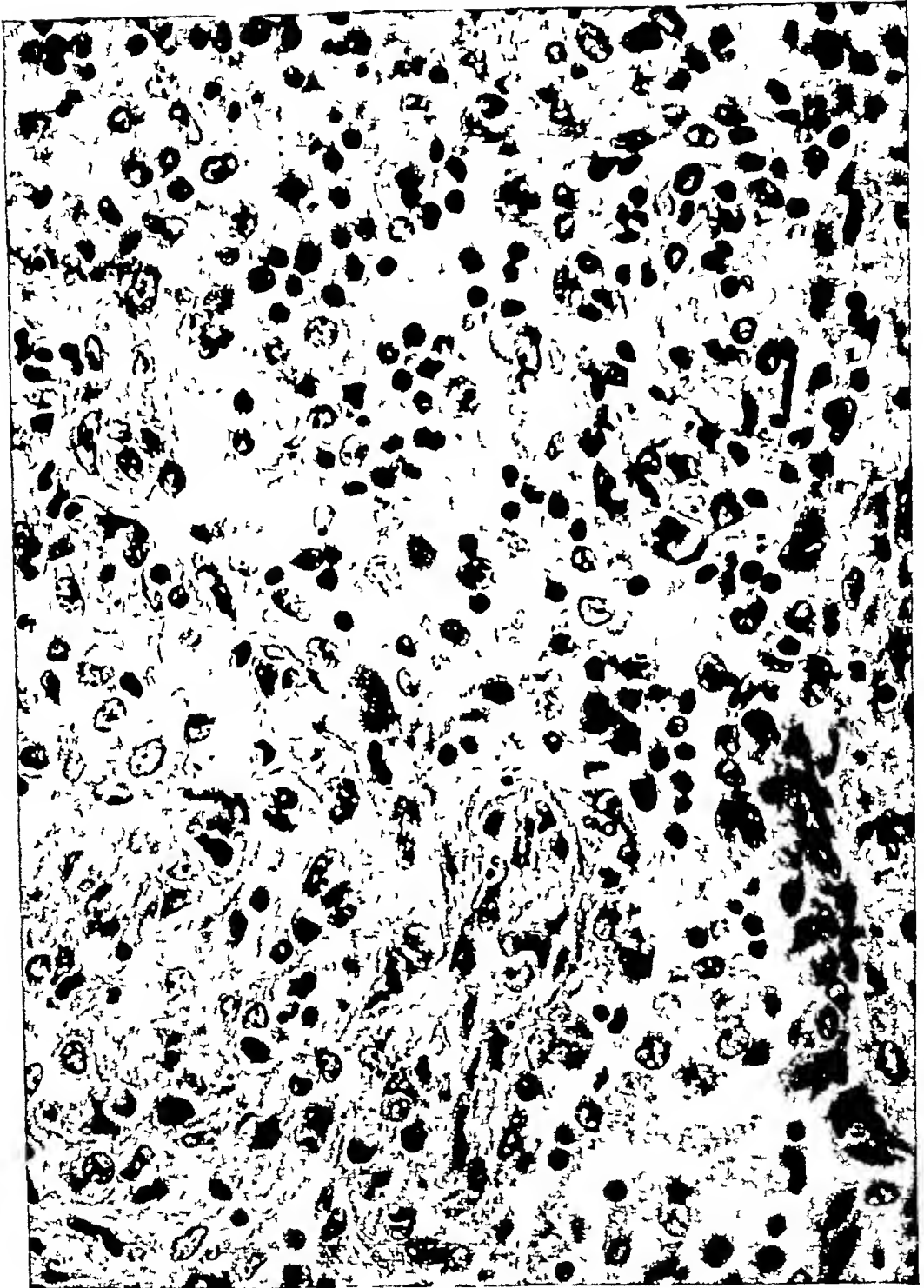


FIG 8

Large splenic blood sinus containing many nucleated red blood cells. The large dark littoral cells contain *Bartonella bacilliformis*. Giemsa stain  $\times 580$ .

2 out of 3 died with typhoid fever, 2 died of a *Salmonella schottmulleri* infection, 3 died with amebic colitis, and in 3 cases the necropsy examination suggested an anaerobic septicemia due to an undetermined organism.



FIG 9

Colony-like masses, each composed of innumerable *Bartonella bacilliformis*, in littoral cells lining a small splenic sinus Giemsa stain  $\times 1275$

In 9 fatal cases, no bacterial or parasitic intercurrent infections were demonstrated. Peripheral collapse occurred in 1 patient who died, and thrombocyto-

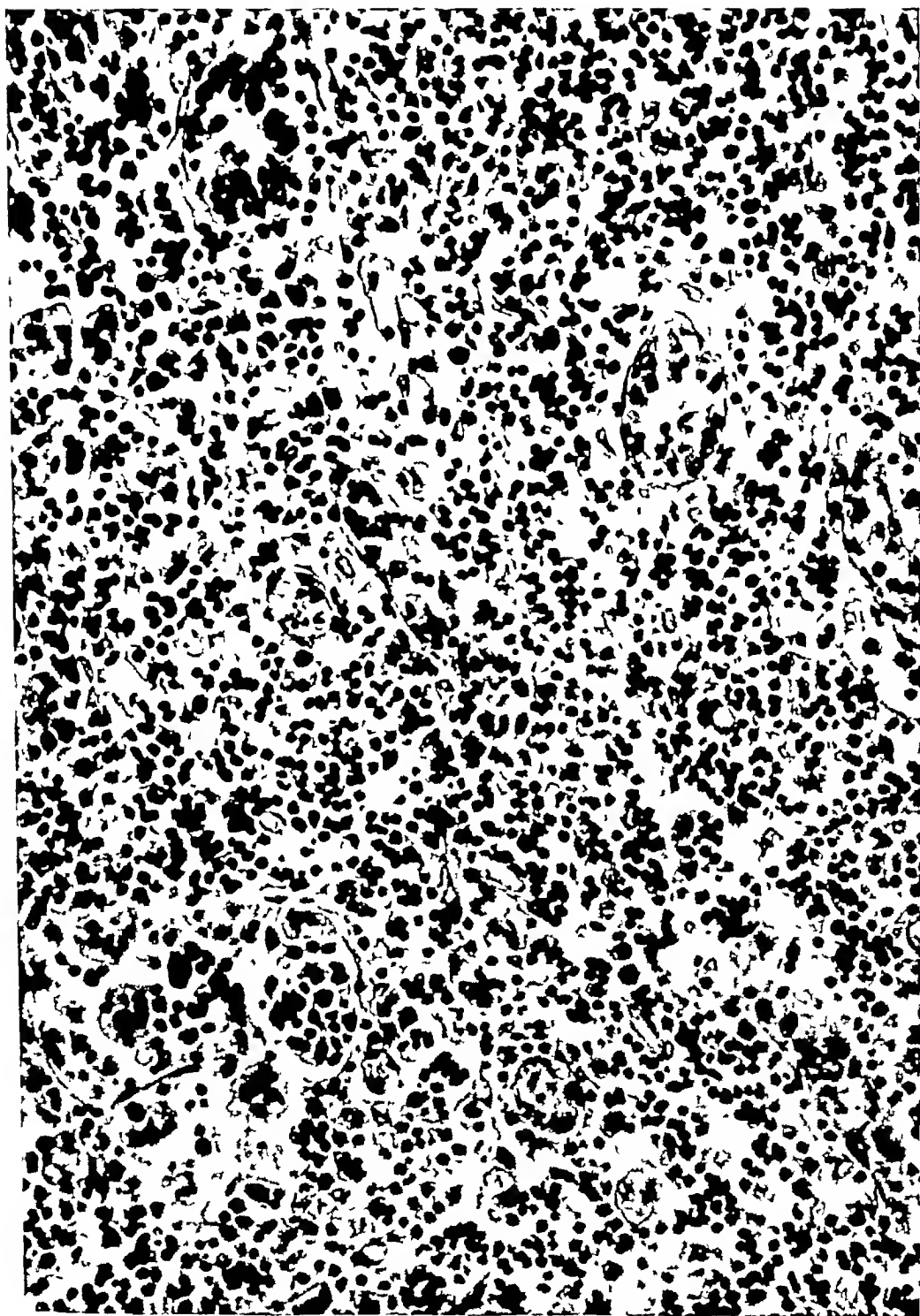


FIG. 10

Bartonella-laden littoral cells lining small blood vessels in a lymph node. Giemsa stain  $\times 335$

penic purpura was the cause of death in two. In 6 others, the cause of death was unknown.

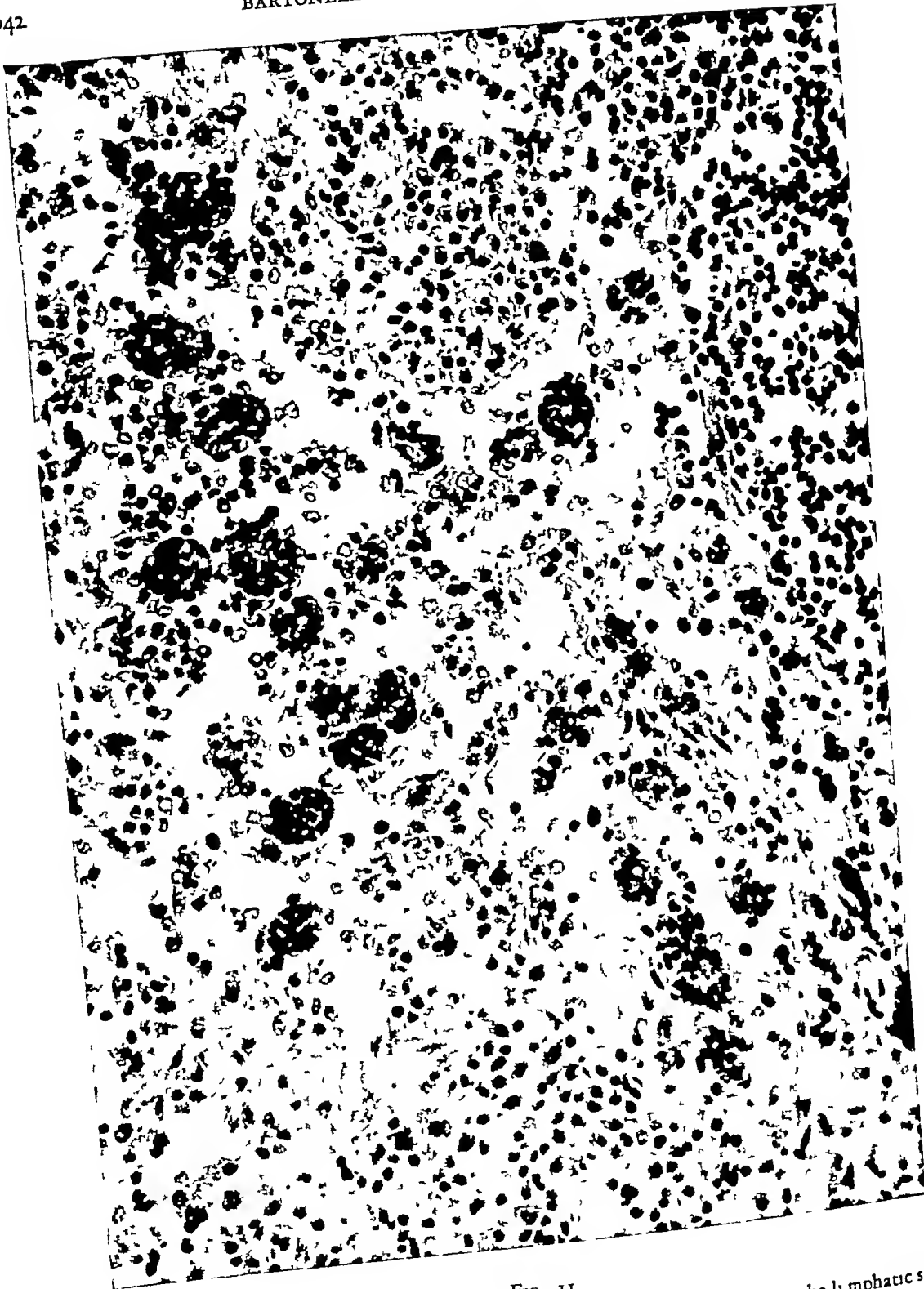


FIG. 11

Section of a lymph node showing large macrophages filled with erythrocytes in the lymphatic sinuses. Bartonellae are seen in the littoral cells of the lymphatic sinuses. Jimenez stain  $\times 325$ .

#### PATHOGENESIS

*Bartonella bacilliformis* anemia is a hemolytic anemia in which the destruction of erythrocytes is dependent on the presence of *Bartonella bacilliformis* on the

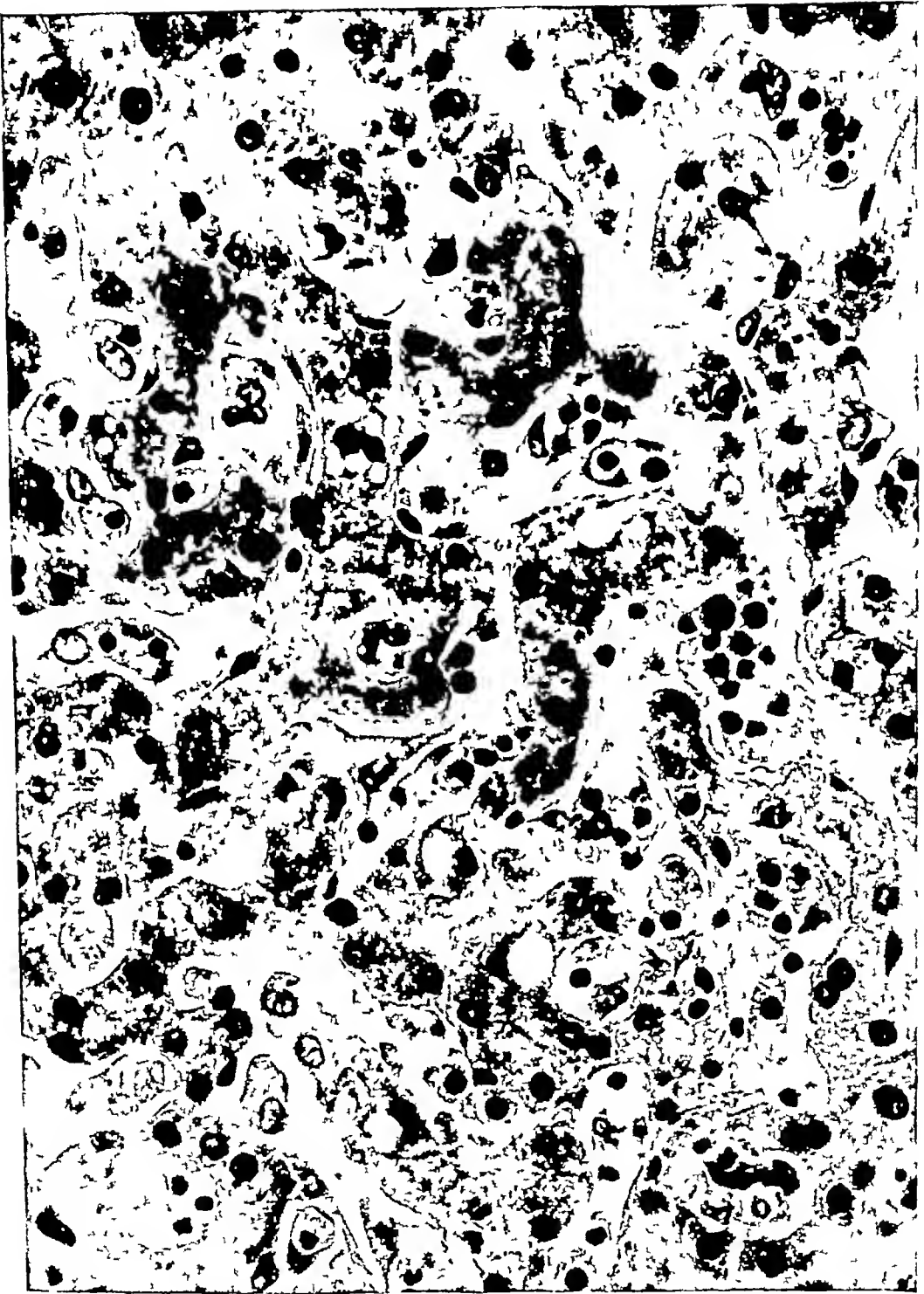


FIG 12.

Section of the liver showing large Kupfer cells, which contain erythrocytes, nucleated red blood cells, and small aggregates of *Bartonella bacilliformis* Giemsa stain  $\times 570$

erythrocytes The rate of destruction of the erythrocytes and the increased bilirubinemia<sup>15</sup> are directly proportional to the number of parasitized erythrocytes<sup>15</sup> The anemia is macrocytic in type with signs of increased blood formation young

granulocytes, polychromatophilia, reticulocytosis and nucleated red cells. Hypochromia is a frequent finding but did not occur in several cases of this series (table 1). There is increased urobilinogen excretion in the feces. Hurtado et al.<sup>15</sup> have shown that the fragility of the erythrocytes is normal in this disease. There are no spherocytes and enlargement of the spleen clinically does not occur in the cases without intercurrent infections.<sup>7, 13</sup> Hemoglobinuria does not occur. An early suggestion was that the bartonella bacilliformis anemia was "Addisonian" anemia. Guzman-Barron<sup>46</sup> demonstrated the presence of Castle's principle in the gastric secretion, and careful hematologic studies<sup>15</sup> have shown that the anemia is normoblastic and not megaloblastic and is accompanied by marked reticulocytosis. Histologically there is evidence of erythrocyte destruction in the reticulo-endothelial system as the Kupffer cells, littoral cells of the spleen and lymph nodes and other phagocytes appear filled with bartonellae and erythrocytes (figs 8-12).

#### PROGNOSIS

Bartonella bacilliformis anemia has a severe prognosis, due mostly to the frequency of intercurrent infection produced by varied organisms from the gastrointestinal tract. The host's defenses, already lowered by the bartonellosis, are overwhelmed by the subsequent infection.

In 30 cases studied in this series, 22 died, a mortality of 73 per cent. Fifteen of these cases had intercurrent infection, and of these, 11 died with septicemia of gastrointestinal origin. In a group of 15 cases without evidence of any concurrent infection, 9 died. In only 2 cases (Cases 3 and 5) could the death be explained by the virulence of the infection and the intensity of the anemia. Case 5 apparently died of a thrombocytopenic purpura with 440,000 erythrocytes. In the remaining 7 fatal cases the intensity of the anemia was not severe enough to explain the fatal course, the severity of the disease apparently being due to the bartonella infection itself.

#### TREATMENT

At present, there is no specific therapeutic agent which acts against the bartonella bacilliformis and the treatment used is largely symptomatic. A great deal of therapeutic effort has been directed toward stimulating the bone marrow. This is of dubious importance in cases without any complication, as the main problem in bartonella bacilliformis anemia is not the formation of red cells but the destruction of erythrocytes. The reticulocytosis is sufficient evidence of regeneration. Case 1 in this series without specific treatment or marrow stimulation showed an extraordinary degree of erythrocytic regeneration, this is seen in every case without intercurrent infection. The sudden transition occurring naturally in the course of the disease with disappearance of the bartonellae from the red cells may give the fallacious impression that a given drug or a treatment is responsible for such change. There is some evidence that liver extracts<sup>15</sup> may accelerate the erythrocyte regeneration.

Blood transfusions appear to be indicated, however, up to the present this method of treatment has been used only occasionally. This is largely due to the

prevailing theory<sup>35</sup> that the crisis of the anemia with the disappearance of the bartonella bacilliformis from the red cells is due to the immunity of the red cells toward the bartonellae. Blood transfusion, according to this theory, will alter the immunity since the donor cells are supposedly more susceptible and so more apt to be parasitized. This is not acceptable, as the erythrocytes have never been known to have immunologic activity. The immunologic changes which occur in the host in the critical stage produce the changes both in the shape and in the clearance of the bartonellae from the erythrocytes. This is supported by the fact that the anemia does not recur in the course of the disease, even if fatal intercurrent infection occurs.<sup>14</sup> Small blood transfusions of 300 to 400 cc. used in a very few cases with severe anemia and thrombocytopenia in my experience have proved to be a life-saving procedure. Unfortunately, larger amounts of blood were not available to test any of the patients included in this series. The other main objective in the treatment of bartonella bacilliformis anemia should be the prevention of intercurrent infection. Penicillin has lately been reported in a small number of cases to produce good results in the treatment of the disease.<sup>47-50</sup> Further studies are necessary to evaluate the action of this drug. In the future, studies should be directed toward the control of the bartonella bacilliformis, as well as the prevention of intercurrent infection.

### DISCUSSION

A severe hemolytic anemia may occur during the invasive stage of Carrión's disease. The pathogenic organism, bartonella bacilliformis, is found in the erythrocytes and on the cells of the reticulo-endothelial system. It has been previously stated that bartonella bacilliformis anemia occurs at any stage of Carrión's disease.<sup>2, 31 et al</sup> However, there are no proven cases in which this anemia has occurred except in the invasive stage of the disease. Anemias which occur later in the pre-eruptive and eruptive stages, were found in a previous study<sup>13</sup> to have a different etiology and different morphologic and clinical characteristics. The anemias without bartonellas in the erythrocytes are due to intercurrent infections, hemorrhages, thrombocytopenic purpura or other causes. Recurrences of bartonella anemia did not occur in these series of patients even in cases with fatal intercurrent infections. This is another important fact against the concept<sup>36</sup> that blood transfusions should not be given to these patients because of the danger of reinvasion of the erythrocyte by bartonellae. On the contrary it seems from this study that these patients will be greatly benefited by repeated blood transfusions from 600 to 1000 cc. as a supportive therapy against the bartonella infection and the anemia. With the disappearance of bartonellae from the erythrocytes, which occurs during the natural course of the bartonella infection, the red cell hemolysis ceases and there is a spontaneous prompt recovery.

The transition period in which the bartonellae suddenly disappear from the erythrocytes in the circulating blood is characterized by a very low resistance of the host to secondary infection. This accounts for a considerable proportion of mortality in this disease, as is illustrated by 11 deaths occurring in 12 septicemic cases. In only 2 cases (Cases 3 and 5) in which the erythrocyte counts were ap-



proximately 5 million/cu mm could death be attributed to the severity of the anemia. In all the other cases the severity of the disease was due to the bartonella infection or to intercurrent infection and could not be related to the intensity of the anemia.

### SUMMARY

*Bartonella bacilliformis* anemia (Oroya fever) is a febrile hemolytic anemia with distinguishing clinical and hematologic characteristics. It occurs as an infrequent clinical form during the invasive stage. The onset is variable with or without chills, followed by a moderate temperature which does not parallel the intensity of the anemia. Hemorrhages, petechial spots, epistaxis may occur and are due to thrombocytopenia. Clouding of the sensorium and delirium are rather uncommon. There is a generalized lymphadenopathy but no splenomegaly.

The anemia is macrocytic and frequently hypochromic with signs of intense blood formation: young granulocytes, polychromatophilia, reticulocytosis and nucleated red cells. The reticulocytosis may increase to 50 per cent. The pathognomonic sign of the disease is the presence of *bartonella bacilliformis* on the erythrocytes. The leukocyte count varies, slight leukocytosis is not uncommon but marked leukocytosis is extremely rare in cases without intercurrent infections. There is a shift of the polymorphonuclear series to the left, characterized by the presence of myeloblasts, myelocytes and metamyelocytes. The anemia is normoblastic and not megaloblastic. It is hemolytic and the destruction of erythrocytes is dependent on the presence of *bartonella bacilliformis* on the erythrocytes. There is no spherocytosis and the fragility of the erythrocytes is normal. Histologically there is evidence of erythrocyte phagocytosis in the cells of the reticulo-endothelial system, the Kupffer cells, the littoral cells of the spleen and the lymph nodes.

The disappearance of the bartonellae from the erythrocytes occurs in a very few days and is called "critical stage" of the anemia. The hematologic changes of this transition are as follows: a change in the shape of the bartonellae from the 'bacilliform' to 'coccoid' form before complete clearance will take place, an increase in the erythrocyte count, reduction in the indirect hyperbilirubinemia to normal, increase in the number of reticulocytes, reversion to normocytosis, lymphocytosis, reappearance of monocytes and eosinophiles, a 'shift to the right' of the polymorphonuclear series. Corresponding with the clearance of *bartonella* from the erythrocytes, the symptoms dependent on the anemia as well as the fever disappear. Clinical improvement, however, may not parallel the clearance of bartonellae because of intercurrent infections or an atypical course of the bartonellosis itself.

*Bartonella bacilliformis* anemia has a very severe prognosis, due largely to the occurrence of intercurrent infection by enteric organisms. The treatment today is largely symptomatic as there is no specific agent against the bartonellae bacilliformis infection.

It is suggested that the prognosis may be improved by the use of adequate blood transfusions and the prophylactic use of antibiotics to control intercurrent in-

fection At present there is no specific agent against bartonella bacilliformis anemia

### CONCLUSIONS

1 Bartonella bacilliformis anemia (Oroya fever) is a type of hemolytic anemia in which the pathogenic organism of the disease is found in the circulating erythrocytes and in the cells of the reticulo-endothelial system

2 Bartonella bacilliformis anemia is macrocytic, frequently hypochromic and occurs only during the invasive stage of Carrion's disease and does not recur in the course of the disease Other anemias which develop later have different clinical and hematologic characteristics

3 The transition period in which the bartonellae suddenly disappear from the erythrocytes depends on immunologic changes of the host toward the bartonella infection

4 The prognosis of bartonella anemia is very grave, as is indicated by a mortality rate of 73 per cent in this series of 30 cases This very severe prognosis is largely due to intercurrent infection which occurred in 50 per cent of the cases studied The most severe prognosis was found in cases with septicemias from enteric organisms, as indicated by 11 deaths out of 12 cases

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## BONE MARROW OF NORMAL GUINEA PIGS

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**M**ARROW aspiration is a well standardized technic for the study of hematopoietic function in the human. The method has been applied to dogs<sup>1</sup> and cats<sup>2</sup> with success. In these animals bone marrow is obtained by aspiration of the iliac crest. The disadvantages of the aspiration method are loss of architecture and mixture of marrow and blood in varying unknown proportions. A great advantage is that frequent, serial examinations showing distinct qualitative differentiation of marrow cells may be obtained.<sup>3</sup> No anesthesia is required, nor is any special aseptic surgical approach necessary.

Epstein and Tompkins<sup>4</sup> reported values obtained both by microscopic section and supravital stain technic. We are presenting data on normal bone marrow as obtained by iliac aspiration, and comparing our values for bone marrow and peripheral blood with those of other investigators.

### MATERIALS AND METHODS

The peripheral blood of 47 normal laboratory guinea pigs, both male and female, of unknown age and of weights varying from 268 to 640 Gm. was studied. Iliac marrow aspiration studies were made on 20 male guinea pigs of this group.

The technic for iliac puncture is essentially the same as that detailed in our study of normal cat bone marrow.<sup>2</sup> The animal is strapped to the table with dorsum presenting and extremities extended. The hair over the sacral area is clipped and the skin sterilized with a suitable antiseptic. Local anesthesia is not required. The anterosuperior border of the iliac crest is outlined with the fingers of one hand. A sterile 20 gage Quincke spinal tap needle about one and a half inches long is inserted through skin and muscle close to the iliac crest. Upon reaching the periosteum the needle is simultaneously rotated and pushed with a boring movement until the needle is firmly imbedded in bone. A sudden "give" is not experienced. The stylet is removed, a dry 20 ml. syringe is attached to the needle and about 0.2 ml. of marrow is aspirated. The needle and the attached syringe is then removed from the animal and a drop expelled on a clean glass slide. Nucleated cell and megakaryocyte counts are made with the technic used in counting the leukocytes of the peripheral blood. Thin films are drawn on several clean glass slides and stained with Wright's stain. Five hundred nucleated cells are counted and the percentages estimated, utilizing the classification of Vogel and his associates.<sup>5</sup> The ilium may be fractured by the procedure, but a repeat aspiration can be made after ten to fourteen days.

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## OBSERVATIONS

The data obtained for the peripheral blood (table 1) are compared with those of other observers (table 2). The similarity indicates that the animals used in the present study were hematologically normal (table 1, Group II).

Bone marrow studies (table 3) indicated a wide range from 481 to 377 thousand cells per cu mm of bone marrow aspirated. The average marrow cell count was 191,200 per cu mm. Similarly, the number of megakaryocytes per chamber varied from 5 to 48 cells. The differential cell count showed a more constant pattern. The

TABLE 1—*The Peripheral Blood of the Normal Guinea Pig*

Animal group number	Number of animals	Weight range Gm	Sex	Hb Gm %	RBC $\times 10^6$ per cu mm	WBC $\times 10^3$ per cu mm	Non Seg-Neutroph	Segmented neutrophiles	Eosinophiles	Basophiles	Mature Lymphocytes	Lymphocytes with Kurloff bodies	Monocytes
I	27	285 to 391	M & F	13.7	5.14	8.00	0.5	44.2	0.3	0.6	52.8	1.0	1.1
II	20	268 to 640	M	14.6	5.22	11.40	0.75	39.2	1.2	0.2	56.3	0.3	1.8
I & II	47	268 to 640	M & F	14.2	5.20	9.46	0.6	42.1	0.6	0.5	54.6	0.7	1.4

TABLE 2—*Peripheral Blood Counts By Different Investigators*

Author	Number of animals	RBC $\times 10^6$ per cu mm	Hb Gms %	WBC $\times 10^3$	P M N	Differential leukocyte count (%)			
						Eosinophiles	Basophiles	Lymphocytes	Monocytes
King and Lucas <sup>6</sup>	9	5.06	—	17.4	31.1	3.5	0.2	63.4	1.8
Scarborough <sup>7</sup>	500±	5.75	—	10.8	41.8	4.8	0.7	45.3	8.4
Wintrobe <sup>8</sup>	4	5.75	14.5	5.5	—	—	—	—	—
Present authors	47	5.2	14.2	9.46	42.7	0.6	0.5	55.3	1.4
Present authors' range		4.5 to 6.9	12.5 to 16.0	5.5 to 22.0	3 to 75	0 to 8	0 to 3	25 to 97	0 to 12

myeloid series constituted 55.4 per cent, 28.3 per cent of which were segmented, and 15.8 per cent nonsegmented 'neutrophiles.' This cell in the guinea pig, when examined with Wright stain, is not neutrophilic but pseudoeosinophilic. The cytoplasmic granules take a pale eosinophilic stain, are rounded and of fairly uniform size. In contrast, the true eosinophile has granules which are large, coarse, and somewhat irregular. Occasional heterophiles were also noted and have been included with the 'neutrophilic' group. Lymphocytes constituted 25.79 per cent of the marrow, 0.4 per cent of which had Kurloff cytoplasmic inclusion bodies.<sup>9</sup> The erythroid series constituted 16.5 per cent of the cells counted.

A comparison of the bone marrow observed by aspiration technic in different

TABLE 3 — *Bone Marrow of Normal Guinea Pigs*

Guinea pig number	Total marrow count $\times 10^3$	Total megakaryocytes per chamber	Granulocytes				Eosinophilic				Lymphocytes		Erythroid						
			Myeloblasts	Myelocytes	Non segs	Segmented	Myelocytes	Non segs	Segmented	Basophiles	Mature	With Kurloff bodies	Plasma cells	Monocytes	Reticulo-endothelial cells	Megaloblasts	Erythroblasts	Normoblasts	Megakaryocytes
56	142	17	0 8	8 4	16 0	21 4	0 6	2 8	4 4	1 6	20 0	1 0	0 2	1 2	0 0	1 8	1 8	17 6	0 4
57	48 1	5	1 1	8 0	15 6	26 6	1 0	1 6	2 2	0 0	32 2	0 4	0 2	1 2	0 2	0 6	1 0	7 9	0 2
58	—	—	1 4	7 8	21 8	21 0	1 8	1 8	1 2	0 4	26 0	0 6	0 0	1 6	0 8	0 8	2 0	11 0	0 0
59	226	31	1 0	6 6	16 6	22 0	1 0	3 0	2 4	1 2	10 8	1 4	0 2	0 4	0 0	1 6	4 4	25 2	0 2
60	90	13	1 0	5 0	17 5	38 7	0 0	2 0	1 3	0 3	22 8	0 2	0 2	0 2	0 0	1 0	2 0	8 8	0 0
61	—	—	0 4	7 2	20 0	33 4	0 2	2 0	0 4	1 2	23 4	0 4	0 2	1 0	0 4	0 6	1 2	8 0	0 0
62	377	38	1 6	7 6	15 3	27 8	1 3	1 8	2 2	0 6	19 0	0 0	0 8	1 1	0 0	2 4	3 7	14 4	0 2
64	150	8	1 0	5 6	17 2	36 4	1 8	2 4	3 4	0 8	22 2	0 0	0 2	0 8	0 0	0 2	1 0	7 0	0 0
65	196	20	1 2	4 6	11 8	32 0	1 2	2 0	3 4	0 6	31 2	0 0	0 6	2 2	0 0	1 0	0 4	7 6	0 7
74	327	30	0 8	4 0	16 2	23 6	0 0	0 6	1 8	1 0	26 8	0 0	0 6	2 2	2 0	1 0	1 4	17 4	0 4
75	146	14	1 2	5 6	16 2	33 0	0 4	1 0	1 8	1 4	24 6	0 4	0 0	2 0	0 0	1 6	2 0	8 6	0 2
76	245	33	1 4	6 2	12 6	36 2	0 4	0 4	3 4	0 2	14 8	0 2	0 2	2 4	0 0	1 8	1 4	18 4	0 0
77	90	8	0 8	4 6	12 0	19 0	0 0	1 8	1 8	0 4	36 0	0 2	0 0	2 4	2 6	0 6	1 6	16 2	0 0
78	108	9	0 6	6 6	9 6	18 2	0 2	0 2	1 2	0 4	48 0	0 0	0 0	1 0	0 4	1 0	2 0	10 6	0 0
79	213	10	0 4	4 2	6 4	23 0	0 0	0 2	0 6	1 2	32 4	0 2	0 8	1 0	0 0	0 6	1 8	28 0	0 0
80	—	—	0 2	5 4	14 2	30 0	0 2	1 0	1 6	1 4	27 2	0 0	2 6	0 4	0 8	1 2	1 8	12 2	0 0
84	270	23	0 4	6 8	28 6	33 6	1 0	1 0	1 4	0 2	19 0	0 6	0 8	1 6	0 2	0 2	0 6	7 6	0 0
87	88	15	0 6	4 0	13 0	32 4	0 4	0 4	1 2	0 2	25 4	0 2	0 2	0 2	0 2	0 6	1 0	19 4	0 0
88	272	48	0 4	6 2	13 8	23 8	0 2	0 2	1 0	0 6	25 0	0 8	0 8	1 0	0 0	0 8	1 8	23 6	0 0
94	259	10	1 0	7 0	20 6	34 0	0 2	0 4	1 8	0 8	22 6	0 6	0 0	2 8	0 4	0 0	1 0	5 8	0 2
Average	191 2	19 5	0 87	6 07	15 8	28 3	0 6	1 3	1 9	0 7	25 42	0 37	0 43	1 4	0 4	1 0	1 7	13 8	0 1

TABLE 4 — *Comparison of Bone Marrow, Man, Dog, Cat and Guinea Pigs*

Bone marrow	Man <sup>a</sup>	Dog <sup>1</sup>	Cat <sup>2</sup>	Guinea pig
Count $\times 10^3$ per cu mm	118 75	144	209 9	191 2
Megakaryocytes per chamber		41 2	20	19 5
Myeloblasts	1 6	0 58	0 82	0 87
Myelocytes—agranulocytic	0 1			
Myelocytes				
Neutrophilic	21 5	3 76	5 22	6 07
Eosinophilic	0 77	0 26	1 1	0 6
Non-segmented				
Neutrophiles	30 2	23 50	38 5	15 8
Eosinophiles	0 39	0 12		1 3
Segmented				
Neutrophiles	34 0	18 50	22 52	28 3
Eosinophiles	0 94	1 56	1 71	1 9
Basophiles	0 07	0 02		0 7
Lymphocytes	8 6	9 84	9 05	25 9
Monocytes		1 20		1 4
Monoblasts		0 14		
Plasma Cells		0 82	0 75	0 43
Reticulum Cells	0 25	0 54	0 02	0 4
Hematogones	3 1	0 44		
Megakaryocytes	0 2			0 1
Megaloblasts	0 14	1 02	0 35	1 0
Erythroblasts	7 1	2 50	1 24	1 7
Normoblasts	22 6	53 18	18 52	13 8

species is shown in table 4. The cell counts of dog, cat and guinea pig agree fairly closely. The guinea pig marrow, however, shows a marked difference in its lymphocytic composition. These elements are significantly increased, more than in any of the other animals studied. The increased lymphocyte content is also seen in the peripheral blood of the guinea pig.

A comparison of bone marrow data with that of Epstein and Tompkins is shown in table 5. It may be observed that the interpretation of bone marrow data varies significantly with varying technics. These differences are probably due to the inclusion of more peripheral blood in aspirated specimens than is obtained in biopsied material.

TABLE 5 — *A Comparison of Technics*

	Microscopic <sup>1</sup> section	Supravital <sup>1</sup>	Aspiration technics, Wright stain
Blasts	4.0	2.8	0.9
Neutrophiles			
Myelocytes	18.2	19.0	6.1
Non-segmented			15.8
Segmented	27.7	16.9	28.3
Eosinophiles			
Myelocytes			0.6
Non-segmented			1.3
Segmented			1.9
Basophiles			0.7
Lymphocytes	10.2	11.3	25.8
Monocytes		11.0	1.4
Plasma cells			0.4
Erythroid cells			
Megaloblasts	3.0	2.4	1.0
Erythroblasts	16.7	6.5	1.7
Normoblasts	17.3	26.6	13.8
Reticulo endothelial cells	1.9	0.7	0.4
Megakaryocytes	1.1	1.6	0.1

## SUMMARY

1. A method is presented describing the technic for obtaining bone marrow from the iliac crest of the guinea pig by aspiration.
2. The cellular constituents in the peripheral blood and bone marrow of normal guinea pigs are presented.
3. Peripheral blood and bone marrow findings in guinea pigs are compared with those of other observers and with other animal species where the aspiration technic was used.

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# SEPARATION OF LEUKOCYTES FROM WHOLE BLOOD BY FLOTATION ON GUM ACACIA

By FRANCES SPEAR, B A , M S

A METHOD of separating leukocytes using serum albumen was described by Vallee, Hughes and Gibson<sup>1</sup> An equally satisfactory and less expensive method has been developed in this laboratory using a gum acacia solution of proper specific gravity, hydrogen ion concentration, and osmotic pressure. Supravital studies of leukocytes separated by this method indicate that cells are viable and apparently undamaged. Preparation of solution

To 10 ml of distilled water in a 100 ml graduate (which has a stopper), 30 grams of gum acacia, and 30 ml water are added. The materials are mixed with a glass rod until solution is complete. Then 0.25 Gm of sodium bicarbonate are added. After mixing well, the glass rod is washed down with distilled water and enough water added to make 90 ml. The graduate is stoppered and heated in a water bath at 50 C for a few days. The solution is agitated at intervals and unstoppered occasionally to permit the escape of carbon dioxide. After a few days, the volume is brought to 100 ml with distilled H<sub>2</sub>O. The pH is tested with a Beckman pH meter. If the pH is below 6.9, heating is continued.

When the solution has reached a pH of 6.9-7.2 through the combined effects of the original amount of sodium bicarbonate and continued heating, pilot tests are run to determine the combination of gum acacia and citrate-saline which will produce the best separation. Eight tenths ml of this gum acacia solution is mixed with 0.30, 0.35, 0.40, 0.45 ml of a 1 per cent sodium citrate in 0.5 per cent sodium chloride. After the solutions are thoroughly mixed, heparinized blood is added in the proportion of 1.5 ml blood to 1.0 ml of gum acacia saline-citrate mixture. With the exception of a few red cells which sink immediately, the blood floats. Tubes are spun at 500 r p m for ten minutes, and 3,000 r p m for thirty minutes. At the end of this time, the red cells are at the bottom of the tube, and the white cells float at the interface of the layers of gum acacia and plasma where they may be easily collected with a capillary pipet.

The collected cells are placed in a small test tube with about twice their volume of 2 per cent citrate and shaken briskly to counteract the agglutination of platelets which was promoted by the colloidal gum acacia solution. To estimate the number of white cells present, the tube may be spun at 2000 r p m for five minutes, the supernatant fluid removed, and the cells resuspended in a measured quantity of 2 per cent sodium citrate. A WBC pipet is used for obtaining the number of cells per ml. The diluent used is a solution of brilliant cresyl blue 0.1 Gm, sodium citrate 3.8 Gm, and water 100 ml. The solution does not hemolyze the red cells which may be present and stains the white cells a pale blue. The total number of WBC present is estimated by multiplying the count per cubic mm  $\times 1000 \times \text{ml of citrate added to cells in the last suspension}$

From the Pathology Laboratory, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Md.

Cells at this point are examined supravitaly using Janus green-neutral red, or pinacyanol. They show Brownian movement and streaming of granules in polymorphonuclear leukocytes to be preserved. An occasional polymorphonuclear cell shows active movement. Lymphocytes show well stained mitochondria and rare neutral red vacuoles.

Six lots of gum acacia have been tried—two lots from E. R. Squibb & Sons and the others from B. R. Elk Co. They are fairly uniform. The specific gravity of the solution used ranged from 1.085 to 1.105. The pH after the addition of sodium bicarbonate was 6.9 to 7.2. Corrections for tonicity, specific gravity, and pH were obtained through the addition of that amount of saline-citrate solution showing the best separations in the pilot tests. The specific gravity of this mixture was 1.079, pH 7.3. Tonicity was measured by the freezing point as indicated on a Beckman thermometer when a sample of the solution was immersed in a Dewar flask filled with cracked ice and salt. The freezing point was  $0.4 \pm$  when the freezing point of distilled water was taken as 0 degrees C.

#### SUMMARY

An inexpensive solution possessing the physiochemical properties necessary for separating leukocytes and erythrocytes on the basis of differences in specific gravity has been described.

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## EDITORIAL

### THE USE OF FOLIC ACID ANTAGONISTS IN ACUTE LEUKEMIA

ACUTE leukemia remains the most important single problem in the field of hematology. Hopes that the disease may ultimately be controlled have recently been revived. It was found by several groups of investigators that folic acid and its conjugates at times seemed to accelerate the leukemic process.<sup>1 2 3</sup> For this reason, the use of antagonists of folic acid suggested itself as a possible therapeutic measure and several of these have been prepared by Subbarow and his associates.<sup>4</sup> By manipulation of the pteroyl glutamic acid molecule, as for example substitution of an  $\text{NH}_2$  group for the OH group in the 4 position of the pteridine ring structure, an anti-folic acid preparation, 4-aminopteroyl glutamic acid or *aminopterin* is produced. Farber and his associates found that of the various folic acid antagonists which they tried in acute leukemia, aminopterin was by far the most potent. In a series of 16 cases of acute and subacute leukemia in children, these workers<sup>5</sup> found that injections of aminopterin regularly resulted in remissions lasting for at least three months in 10 of the cases. The remissions were characterized not only by great subjective and objective improvement but by improvement in red count and in platelets, a virtual disappearance of primitive white cells from the blood, and a considerable improvement in the appearance of the marrow.

Farber's results, which were obtained in children, have not as yet been duplicated in adults. Personal communications from various centers where aminopterin and related drugs are being used indicate that good results have often been disappointing and that reactions such as hemorrhage, aplastic anemia, etc. are common. However, in our own series of 16 adults with acute and subacute leukemia, 4 patients with acute or subacute leukemia have developed clear-cut remissions characterized by subjective improvement, rise in red cells and platelets, disappearance of blast forms from the blood and a distinct improvement of the marrow picture. We have also had a clear-cut remission in one childhood case of two treated. Although these results may be considered as coincidental by some observers, the remission rate in Farber's series of 10 of 16 consecutive cases is far beyond the spontaneous rate of remission. We have been impressed, therefore, both in our own small series of cases and through examination of Farber's data, that the factor of spontaneous remission can be completely ruled out. If this is the case, then we may indeed be approaching the future control by chemotherapeutic means of what is now a practically hopeless disorder. The therapeutic effect of anti-pteroylglutamic acid preparations indicates that PGA may be one of the materials concerned in the growth processes of the white cells of leukemia. What is even more likely is that other enzymes more important than PGA are necessary for leukocytic growth. When these are found, anti-enzymes may then be discovered. Certainly further work along these lines is indicated.

Aminopterin and related drugs must be used with great caution for they can

severe reactions of the mucous membranes and, in large doses, of the marrow as well. These chemicals should by no means be considered as cures, all they apparently do, even in the most favorable cases, is to keep the "flame" of leukemia under temporary control. However, the knowledge that even a little something can be accomplished in acute leukemia is very heartening news indeed, and a great stimulus for continued investigation.

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# ABSTRACTS

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## ANEMIA

EFFECT OF XANTHOPTERIN ON CELL PROLIFERATION IN BONE MARROW CULTURES *E. R. Norris and J. J. Majnarich* From the Department of Biochemistry, University of Washington, Seattle, Washington  
*Am J Physiol* 152: 175-178, 1948

Since 1936, Xanthopterin has been reported as curative of certain (nutritional) anemias and leukopenias in rats and fish. In 1940, xanthopterin was isolated from liver extract used in treating pernicious anemia. The present report concerns the effect of xanthopterin on erythropoiesis and leukopoiesis in bone marrow cultures *in vitro*.

The authors suspended bone marrow (rabbit, beef, sheep, rat, cat) in Tyrode's solution, added specified supplements (Tyrode's solution, folic acid, xanthopterin, normal human serum), and counted the various blood cells after three and six hours of incubation of the resultant mixture. They found that xanthopterin caused an increase in the rate of cellular proliferation of white cells, reticulocytes, and red cells, and that such an effect was optimal when the concentration of xanthopterin was about five micrograms per milliliter of suspension. The results obtained with xanthopterin and with normal human serum were of the same order of magnitude and were prompt. Folic acid, on the other hand, had no enhancing effect on proliferation of cells in these suspensions, suggesting, as the authors subsequently point out, that folic acid as such is unavailable to the marrow cells for growth. In a subsequent report dealing with nutritional anemia in rats, folic acid similarly was found to have an effect only after a lag period of several days, whereas the hematopoietic effect of xanthopterin was immediate (*Am J Physiol* 152: 179-182, 1948)  
S. E.

XANTHOPTERIN IN RAT ANEMIA PRODUCED BY SULFATHIAZOLE *E. R. Norris and J. J. Majnarich* From the Department of Biochemistry, University of Washington, Seattle, Washington  
*Am J Physiol* 152: 179-182, 1948

Anemia was produced in rats by feeding a purified diet augmented with 1 per cent sulfathiazole. The rats were then divided into three groups (with corresponding controls), and treated with xanthopterin or pteroylglutamic (folic) acid. The effect on the blood values was studied.

*Group 1* These rats received 100 micrograms of xanthopterin daily for five days. There was an immediate and marked rise in red cells, hemoglobin, and hematocrit, which did not occur in the controls. A peak was reached, and then the blood values began to fall rapidly to their pretreatment ranges.

*Group 2* These rats received single injections of xanthopterin (25, 50, or 100 micrograms) or synthetic pteroylglutamic acid (100 micrograms). The control group, which received no injections, died in seven days. Xanthopterin-injected rats showed a prompt rise in red cells, reticulocytes, white cells, and hematocrit, the rise being greater the greater the dose of xanthopterin. Rats receiving pteroylglutamic acid also showed a rapid rise in all blood elements, but there was a delay period of three to five days before this rise began.

*Group 3* These rats received, by a single injection, either 100 micrograms of xanthopterin, or 300 micrograms of pteroylglutamic acid (this amount contains the same weight of pteridine as 100 micrograms of xanthopterin). Again, there was a rise in all blood values, but whereas the rise with xanthopterin was immediate, that with pteroylglutamic acid followed a lag period of one to three days.

It is suggested as a result of these experiments and similar results *in vitro* with bone marrow culture

(*Am J Physiol* 152 175-178, 1948), that xanthopterin can be utilized directly by the bone marrow in hematopoiesis, whereas pteroylglutamic acid cannot be utilized as such, but must be altered in form before it can affect hematopoiesis. It is strange that, in contrast, fragmentary experiences with xanthopterin in pernicious anemia suggested that it was of no value in the dosages employed (*South M J* 40 46-55, 1947), although, as is well known, pteroylglutamic acid is highly effective

SE

OBSERVATIONS ON THE ANTIANEMIC PROPERTIES OF VITAMIN B<sub>12</sub> T D Spies, R E Stone, and T Aramburu From Northwestern University at Hillman Hospital, Birmingham, Alabama *South M J* 41 522-523, 1948

Crystalline vitamin B<sub>12</sub> was administered by single intramuscular injection to two patients with pernicious anemia, two patients with nutritional macrocytic anemia, and one patient with nontropical sprue. Five of the patients received six micrograms of the material, the sixth, 15 micrograms. Within three to five days after the injection, the patients felt better and stronger, the soreness and burning of the tongue disappeared, and there was objective clinical and hematological improvement. Reticulocytosis (twelve to twenty-two per cent) occurred by the fifth to ninth day, and a rise in red count and hemoglobin followed. Although bone marrow examinations were done, no details are given in this brief report. There was also no extended followup.

This report confirms similar experiences in three patients with pernicious anemia following single injection of three to 150 micrograms of vitamin B<sub>12</sub> (West, *Science* 107 398, 1948). It is possible that this material isolated from potent liver extracts, may be the essential substance required for pernicious anemia.

SE

OBSERVATIONS ON THE HEMOPOIETIC RESPONSE OF PERSONS WITH TROPICAL SPRUE TO VITAMIN B<sub>12</sub> T D Spies, G G Lopez, F Milanes, R L Tola, and B Culver From the Northwestern University Departments of Nutrition and Metabolism, Calixta Garcia Hospital, Havana, Cuba *South M J* 41 523-525, 1948

In two patients with typical tropical sprue, the administration of eight micrograms of crystalline vitamin B<sub>12</sub> intramuscularly was followed within three days by marked subjective and objective improvement (mouth and tongue symptoms relieved, strength increased), as well as by a rise in the various blood elements, with reticulocyte peak in each case on the eighth day. No data beyond the eighth day of treatment are given.

SE

HIGH SERUM ACETYLCHOLINE CONCENTRATIONS IN PERNICIOUS ANEMIA AND THEIR REDUCTION BY EFFECTIVE THERAPY J E Davis From the Departments of Physiology and Pharmacology, University of Arkansas School of Medicine *Am J Dig Dis* 15 52-55, 1948

In dogs, the continued injection of acetylcholine produces a hyperchromic anemia which responds to antipernicious-anemia therapy, and the injection of derivatives of acetylcholine in dogs may produce central nervous system changes resembling those found in human pernicious anemia. The author, having developed a bio-assay method for the determination of the amount of acetylcholine in serum, measured the serum acetylcholine in five patients with relapsed pernicious anemia, two patients with remitted pernicious anemia, six normal individuals, and six individuals with secondary anemias (lymphosarcoma, leukemia, sickle-cell disease). The following results were obtained. It was found that the normal level of acetylcholine in serum ranged from 6.6 to 8.2 micrograms per 100 cc of serum, the level in secondary anemias was the same, but in relapsed pernicious anemia, levels of 15 to 33 micrograms per 100 cc were obtained. When specific treatment of these latter patients was instituted (liver extract, stomach extract, or pteroylglutamic acid), there was a marked reduction in the serum acetylcholine to normal levels within four to seven days.

It is speculative, whether the aberration in serum acetylcholine is fundamentally related to the occurrence of pernicious anemia. The author suggests the possibility that something produces an excess of acetylcholine in the serum, which in turn depresses or arrests erythropoiesis within the marrow, resulting in the disease. Actually, he points out, the serum cholinesterase in pernicious anemia is normal or only

slightly decreased, and in remitting pernicious anemia the cholinesterase may actually fall even further, whereas in normal and leukemic individuals, pteroylglutamic acid causes a rise in serum cholinesterase. It is possible, therefore, that perhaps the effect of antipernicious-anemia medicaments is to raise the level of cholinesterase in the blood cells rather than the serum (for this there is some evidence), that a fall in acetylcholine results from this, and that, with the fall, erythropoiesis reverts toward normal. These speculations are still tentative and, of course, do not suggest the basic cause for the alterations in the serum levels of acetylcholine or cholinesterase.

S.E

EFFECT OF FOLIC ACID AND LIVER EXTRACT ON SERUM AND RED CELL CHOLINESTERASE ACTIVITY *A M Kunkel, S Krop, and W C Wescoe* From the Pharmacology Section, Medical Division, Army Chemical Center, Maryland *Am J Physiol* 152 309-313, 1948

A diminution in the blood serum cholinesterase (ChE) has recently been reported in pernicious anemia, and it has been found that the administration of liver extract or pteroylglutamic acid in such cases was followed by a rise in the serum ChE, concomitant with the clinical and hematological improvement following such treatment. It has been suggested, therefore, that the role of liver extract or pteroylglutamic acid may be to cause an increase in serum ChE, which in turn is followed by an improvement in the anemia. The purpose of the present article was to test whether these materials actually cause a rise in serum ChE in vitro or in vivo.

Several experiments were performed

1. Various amounts of pteroylglutamic acid and liver extract were added to normal human and dog plasma in vitro, and the mixture incubated at body temperature. The ChE levels of the plasma remained unchanged.

2. Dog plasma depleted of ChE by the use of diisopropyl-fluorophosphate (DFP), which irreversibly inactivates ChE, was similarly incubated in vitro with folic acid and liver extract. There was no rise in the ChE level.

3. An attempt was made to produce macrocytic anemia (with the accompanying decrease in ChE) in dogs by administration of acetylcholine and physostigmine. No anemia could be so produced, and, furthermore, there were no deviations of serum ChE levels in these dogs during this regimen, or after the addition of liver extract or pteroylglutamic acid.

4. Four dogs were given DFP, so that their plasma ChE level was much reduced. Two were followed without further therapy, to a third, 2 mg of pteroylglutamic acid were administered daily, and to the fourth, 2 units of liver extract daily. The recovery of plasma ChE to normal values was identical in all four dogs.

These data negate the suggestion that it is possible to cause an increase in the amount of serum ChE by the use of the antipernicious-anemia drugs, under the conditions of the experiments. They do not support the hypothesis that an acetylcholine-cholinesterase mechanism is concerned in erythropoiesis. Previous work had already shown that plasma ChE rises (in states of depletion) at a rate similar to the rate of regeneration of other plasma proteins. It seems probable, therefore, as the authors suggest, that the rise of plasma ChE in the successful treatment of pernicious anemia is the result, rather than the cause, of recovery.

S E

DEVELOPMENT OF APLASTIC ANEMIA DURING THE USE OF STREPTOMYCIN. REPORT OF TWO CASES *V F Deyke and J B Wallace* From Fitzsimmons General Hospital, U S A *J A M A* 136 1098, 1948

Streptomycin, according to the authors, has been used in some 900 patients in the literature. Of these patients, eight developed mild, self-limiting leukopenia, and one developed agranulocytosis, the outcome of which was unstated. Of 400 additional patients who received streptomycin in the authors' hospital, two developed aplastic anemia on the 79th and 95th days of treatment, respectively. In the absence of other etiologic agents for marrow involvement (other drugs, disseminated tuberculosis), the aplasia had to be attributed to the streptomycin.

The first patient, a 46 year old man with moderately advanced tuberculosis, received two grams of streptomycin daily for 79 days when his blood, previously normal, showed anemia, leukopenia, and neutropenia. Although the drug was discontinued, and various supportive measures used, the pancytopenia



topenia progressed, and the patient died 19 days later. Prior to death the white cell count was 800, with two per cent neutrophils (16 per cu mm), and red cells and platelets were much reduced. The marrow at autopsy was hypoplastic.

The second patient, a 27 year old man with moderately advanced tuberculosis, also received 2 grams of streptomycin daily, on the 95th day, pancytopenia was noted and progressed despite discontinuation of the medication. Marrow punctures showed hypocellularity of the marrow. The patient was still living at the time of the report.

S E

**IDIOPATHIC HYPOPLASTIC ANAEMIA WITH BONE MARROW HYPERPLASIA** *E S Mills* From the Departments of Medicine, McGill University and the Montreal General Hospital, Montreal, Quebec. *Canad M A J* 57 227-232, 1947

Three fatal cases of idiopathic anemia are described and autopsy findings in two of the cases are included. All three patients were males and each presented variants from the generally accepted pattern of so-called aplastic or hypoplastic anemia. One patient, who received 196 transfusions of 500 cc each, developed generalized hemosiderosis and brownish pigmentation of the skin. Remission occurred in this case after 12 years of illness, but death occurred soon thereafter as a result of pneumococcal infection.

Erythroid hyperplasia of the bone marrow in two of the cases is stressed and it is suggested that there is a maturation arrest in this disease due to deficiency of a factor other than that which is lacking in pernicious anemia.

In the author's interesting discussion of these cases is included a statement that the life span of a red cell is probably less than forty days. This statement should be challenged in view of the conclusive evidence from many laboratories that the life span of normal human erythrocytes is about 120 days.

L E Y

**CLINICAL APPLICATIONS OF BONE MARROW EXAMINATION IN CHILDHOOD** *I J Wolman, and B Dickstein* From the Children's Hospital of Philadelphia, the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. *Am J M Sc* 214 677-687, 1947

The authors have summarized the variety of information to be gained from sternal puncture. The methods of marrow aspiration in infancy and childhood are discussed, as well as normal and pathological cytologic findings. Attention is called to certain bacterial and protozoal infections and metabolic disorders in which diagnosis may be made by sternal aspiration.

C A F

**TROPICAL MACROCYTIC ANAEMIA AND NUTRITIONAL MACROCYTIC ANAEMIA** *H C Trowell* From the Mulago Hospital Medical School, Kampala, Uganda, South African. *J M Sc* 12 21-32, 1947

The response of sixty-three patients with severe anemia to therapy were studied by the authors. Of these, forty-three were macrocytic, twelve normocytic and two microcytic. Improvement following administration of iron and liver extracts was variable and not related to the red cell size. Crude liver extracts were more effective than highly concentrated liver, similar to the cases reported by Watson and Castle (*Am J M Sc* 221 514). The incidence of hookworm infestation (eighty per cent) was higher among these patients than in the general hospital admissions (forty-six per cent). The authors felt that tropical anemia differs from pernicious anemia in not showing a true megaloblastic anemia and in its response to liver therapy. They believe it represents several deficiencies rather than deficiency of the intrinsic factor.

C A F

**RELATION BETWEEN SERUM PROTEIN AND HAEMOGLOBIN IN 324 INDIAN SOLDIERS** *H Lehmann* From the No. 1 Detachment, Anaemia Investigation Team, General Headquarters, India. *Brit J Exper Path* 28 377-384, 1947

Previous surveys of the nutritional status of Indian troops indicated that there was more of a correlation between hemoglobin and serum protein levels than had been reported in similar studies in Europe and North America. Although several methods were used for determining serum protein, preference was given to the copper sulphate gravity method of Phillips et al. In these cases the hemoglobin determina-

tion seemed to be a more sensitive test for malnutrition than the determination of serum protein. In macrocytic anemias the dietary deficiency produces low serum protein levels. But in hypochromic or normochromic anemias due to a dietary deficiency in iron, the serum protein is not necessarily lowered.

O P J

**NORMAL RED CELL SURVIVAL IN MEN AND WOMEN** S T Callender, E O Powell and L J Watts From the Nuffield Department of Clinical Medicine, Oxford. *J Path and Bact* 59 519-532, 1947

Blood was removed from four young women and two male medical students who belonged to group A and were Rh-positive. It was replaced by equal volumes of group O blood. By differential agglutination it was determined that the survival time was about 90-100 days in women and 110-120 days in men. The curve of decay of transfused cells was linear for men and slightly curved for women. Apparently blood destruction is greater in women. The estimated figure of 400 cc per month is much larger than that lost by menstruation alone.

O P J

## HEMOLYTIC ANEMIA

**PHYSIOLOGIC ICTERUS OF THE NEWBORN** A Loewy and L W Freeman From the Department of Physiology, University of Chicago, Chicago, Illinois. *Am J Physiol* 152 205-209, 1948

This paper suggests that the fat diet of the newborn infant plays a role in the rapid destruction of erythrocytes which gives rise to physiologic icterus of the newborn. The authors compared the serum bilirubin in cord blood at birth, with that in venous blood on the fifth day of life, in three groups of newborn infants: those on a routine diet (3.6 per cent fat), those on a low-fat diet (1.8 per cent), and those on a fat-free diet (0.03 per cent). (An attempt to evaluate a fourth group, on a 5.5 per cent fat diet, was unsuccessful because of refusal of feedings.) The average serum bilirubin at birth was 1.36 mg per cent. On the fifth day of life, high-fat babies showed 5.4 mg per cent of bilirubin, low-fat 4.2 mg per cent, and fat-free, 3.09 mg per cent.

Although these results are striking, they must be considered suggestive rather than conclusive, chiefly because of the small number of cases. Thus, there were ten children in the first group (3.6 per cent fat), although six showed a rise in serum bilirubin, four showed either no change or an actual fall. Of twelve children on the low-fat diet (1.8 per cent fat), eight showed the rise, but four showed no rise or a fall, and of nine children on the fat-free diet, six showed a rise, with the other three showing a definite fall. The procedure of averaging such small groups, as the authors themselves point out, may lead to erroneous results. There is, however, a trend, and it is not unlikely that sudden exposure of the newborn to a relatively high fat diet (fat is thought to be barred by the placenta) plays some role in physiologic icterus. That it is not the only factor is obvious.

S E

**A NEW TYPE OF HEREDITARY HEMOLYTIC JAUNDICE WITHOUT SPHEROCYTOSIS** R L Haden From the Cleveland Clinic, Cleveland, Ohio. *Am J M Sc* 214 255-259, 1947

The author reports eight patients in two families with hemolytic anemia. The anemia was macrocytic, normochromic, accompanied by reticulocytosis, bilirubinemia, and splenomegaly. Fragility tests were normal and no spherocytes were seen. In two patients of one family, hemoglobinemia and hemolysis of erythrocytes on incubation was observed, but the Donath Landsteiner and acid hemolysis tests were negative. In the second family, stippled cells were consistently observed. The two groups show significant differences and neither fit into conventionally recognized types of hemolytic anemia. The author suggests that these are instances of congenital stromal defects of the erythrocytes.

C A F

**ICTERE CHRONIQUE JUVENILE NON HEMOLYTIQUE AVEC REACTION DE HIJMAN VAN DEN BERGH INDIRECTE (DISREGULATION DU METABOLISME DES PIGMENTS BILIAIRES) (CHRONIC JUVENILE NONHAEMOLYTIC JAUNDICE WITH INDIRECT HIJMAN VAN DEN BERGH REACTION (FAULTY BILE PIGMENT REGULATION))** *J Carols et A Paraf* *Revue d'Hematologie* 267-282, 1947

The authors report the occurrence of jaundice in an eighteen year old boy, otherwise in perfect health.

This jaundice had existed since he was three years old. It consisted of a frank chronic mucocutaneous coloration, with occasional slight remissions. There had been no similar case in the family. The blood count was normal. Hemolysis of the red cells started in 0.48 per cent saline and was complete at 0.4 per cent. Rouleaux formation was normal. The average red cell diameter was  $7.5\mu$  and there was no spherocytosis. The blood group was O, Rh positive. The myelogram was normal (13 per cent erythroblasts). There were no bile pigments or bile salts in the urine. Urinary urobilin was 0.3 mg per litre, and 0.73 over twenty-four hours. Fecal urobilinogen (Watson method), 23 mg. Indirect blood bilirubin, 18.75 units, 1 c, 98.4 mg, and two days later, 24.60 units, 1 c, 98.4 mg. Cholemia was 10 mg per litre of serum. Finally, the urinary porphyrins were 40 per 1000. All liver function tests gave normal results. The authors discuss the nosological significance of this very definite observation and compare it with that of Dameshek and Singer (*Arch Int Med* 67:259, 1941) and of the hereditary jaundice of the rat studied by Mallay and Lowenstein. They think it is a nonhemolytic jaundice caused by an inborn error of the metabolism of the bile pigments with a kind of regurgitation of the indirect bilirubin.

J P S

**RAPID TEST FOR THE DEMONSTRATION OF SICKLE CELLS AND ITS CLINICAL SIGNIFICANCE** K. Singer and S. Robin. From the Department of Hematologic Research, Michael Reese Hospital, Chicago, Illinois. *J A M A* 136:1021-1025, 1948.

Sickling of susceptible red cells takes place only if the hemoglobin within the red cell is in the form of reduced hemoglobin. Oxygenating the reduced hemoglobin causes a reversal of the sickled cell to a normal biconcave disc. The various tests for sickling depend, correspondingly, upon reduction of the contained hemoglobin, and their speed and efficiency may be expected to vary directly (other things being equal) with their ability to cause this reduction.

The present authors utilize a culture of nonpathogenic bacteria (*B. subtilis*) to eliminate the oxygen in a blood sample, and thereby produce favorable and necessary conditions for sickling to occur. A drop of blood is placed upon a coverslip, a drop of the bacterial culture added to it, and the coverslip is placed upon a glass slide and ringed with paraffin. The preparation is then incubated at  $37^{\circ}\text{C}$  for five minutes and examined; if negative, it is incubated for an additional ten minutes and re-examined. Most positive tests appear within five minutes, and all within fifteen minutes, so that, according to the authors, those that are negative after fifteen minutes of incubation may be considered to be truly negative. The test does not discriminate between the severe sickle-cell anemia and the milder sickle-cell trait, since in both conditions the same abnormality of the red cell, a tendency to sickle under conditions of anoxia, is present.

This is the simplest of the reliable tests for the detection of sickling yet reported. The maintenance of an active culture of *B. subtilis* is discussed by the authors. Once started from agar slants, such a culture is carried with ease by day-to-day reinoculation of media, but refreshment from agar cultures at intervals is required. The speed of the results is of course a great asset.

S E

**THE BASOPHILIC PROPERTY OF THE IRON-CONTAINING GRANULES IN SIDEROCYTES** J. V. Dacie and I. Doria. From the Department of Pathology, British Postgraduate Medical School, London. *J Path and Bact* 59:684-686, 1947.

It has been known that some red corpuscles from patients with severe hemolytic anemia following splenectomy contain basophilic iron-positive granules. The present authors have shown that not all of the basophilic Romanowsky-positive bodies contain iron. This may be due to the fact that the iron content is too small for detection. Negative reactions for iron of basophilic stippling and Howell-Jolly bodies were obtained. In marrow from these patients, siderotic granules were found in nucleated red cells and in some instances they showed a perinuclear arrangement. It has been suggested that their appearance parallels that of hemoglobin because siderotic granules were absent from the more primitive nonhemoglobinized red cell precursor.

O P J

**PATERSON-PLUMMER-VINSON SYNDROME IN A CASE OF FAMILIAL ACHOLURIC JAUNDICE** L. Rau. *Proc Roy Soc Med* 40:271-272, 1947.

The authors present the coincidence, in a patient with familial spherocytic hemolytic anemia,

superimposed chronic iron deficiency (lip fissures, atrophic glossitis, koilonychia, hypochromic anemia) which included dysphagia and, on x-ray, spasm of the esophagus. The hypochromia responded to iron therapy, but there were no changes in the glossitis, nail changes, or esophageal picture, and reticulocytosis and increase in hypotonic fragility of the red cells persisted after the blood picture had lost its hypochromia.

The occurrence of both disorders in one patient must be very rare.

S E

THE MOTHER-CHILD INCOMPATIBILITY PROBLEM IN RELATION TO THE NERVOUS SEQUELAE OF HEMOLYTIC DISEASE OF THE NEWBORN *D F Cappell Brain 70 486-494, 1947*

Kernicterus, the staining of certain parts of the brain by bilirubin in congenital hemolytic anemia, may be followed by permanent neurologic and mental changes in the surviving child. The author of this review presents the current concepts of erythroblastosis fetalis, and then discusses the possible relation of Rh incompatibility between child and mother, and subsequent mental difficulties in general.

Three forms of erythroblastosis are recognized: hydrops fetalis (with the child usually stillborn), icterus gravis neonatorum, and congenital hemolytic anemia. Kernicterus was noted only in those children who survived beyond the first few days of life; thus, in the stillborn with hydrops fetalis, kernicterus has not yet had time to occur, but if the newborn child with hydrops or severe icterus dies several days later, kernicterus is present. Kernicterus was not found in mild hemolytic anemias without jaundice, but only if jaundice was present. It was not possible to correlate the occurrence of kernicterus with the type of antibody found, the abnormality being found no matter which antibody predominated (agglutinins, blocking antibodies). In all infants surviving icterus gravis, evidence of nervous damage in later life was present in some 17 per cent of the cases.

The author does not agree that agglutination thrombi of red cells are responsible for the neurologic abnormalities (death and staining of nerve cells), and cannot suggest the reason for selective involvement of the cortex and basal ganglia.

With respect to the general question of the possible relation of mental deficiency in general to icterus neonatorum, the author reports on 200 mental defectives. He was unable to find a mother-child Rh incompatibility in various types of nonspecific mental defects as compared to cases of Mongolism, and as compared to normal children. This contradicts the work of Yannet and Lieberman (*J A M A* 130 335, 1946), who found that, in a group of Rh positive mental defectives, there were twice as many Rh negative mothers as would be expected on a random basis, but Cappell points out how their figures were inadvertently weighted. It is generally agreed that there is a relationship between the occurrence of kernicterus and the severity of blood destruction (*V C Vaughn, J Ped* 29 462, 1946), and that mental defects occur only if kernicterus has occurred (*M Creak, Arch Dis Childhood* 22 180, 1947).

S E

VISCERAL LEISHMANIASIS COMPLICATED BY SEVERE ANEMIA—IMPROVEMENT FOLLOWING SPLENECTOMY *J H Birchenal, R F Bowers, and T A Haedicke From the Medical and Surgical Services, Walter Reed General Hospital, Washington, D C Am J Trop Med 2 699-709, 1947*

An unusual case of kala azar refractory to a variety of agents and several courses of therapy is described, occurring in a twenty-three year old Negro soldier. In addition, a severe anemia with reticulocytosis and hyperbilirubinemia was present along with a leukopenia. Fifty-one liters of blood were given over a period of twenty-two months. Following removal of the spleen weighing 3050 gms., the patient made a recovery from the anemia and leukopenia and had no further symptoms of leishmaniasis. The organisms were recovered from the spleen at the time of the operation by hamster injection. The authors are anxious not to give the impression that splenectomy is indicated or necessary in most cases of kala azar, although in this instance of refractory disease complicated by hemolytic anemia it was particularly effective.

R S E

# BLOOD COAGULATION AND HEMORRHAGIC DISEASE

QUANTITATIVE STUDIES ON THE COMPARATIVE ACTIVITY OF CALCIUM AND CHEMICALLY RELATED IONS ON THE COAGULATION OF BLOOD *M Stefanski and A J Quirk From the Department of Biochemistry Marquette University School of Medicine Milwaukee, Wisconsin Am J Physiol* 155 389-396 1948

Amberlite IR-100, a phenol formaldehyde resin, is capable of removing calcium from blood serum without otherwise producing any changes in the blood. The authors utilized this material to study the effects of various concentrations of calcium, strontium, magnesium, and barium on blood coagulation. Blood was obtained from man, dogs, and rabbits, decalcified with the Amberlite, and stored on ice. The coagulation time of the decalcified blood was determined after addition of specified concentrations and amounts of the various ions. Thromboplastin was reduced to a minimum by the use of silicon-coated needles, syringes, and glassware.

It was found that coagulation did not occur until the concentration of calcium chloride in the blood was of the order of  $0.0015\text{ M}$ . This is the actual level of calcium in the blood in the human body. Once this level of calcium was reached, there was a relatively wide range of concentrations in which coagulation occurred normally. A delay in coagulation did not occur until the concentration of calcium was reduced below one-half of normal. Above a calcium level of  $0.004\text{ M}$ , the coagulation time of the blood became more and more prolonged.

Strontium behaved similarly to calcium in its ability to cause coagulation of decalcified blood, but was much weaker. Magnesium was found incapable of causing coagulation, except in the presence of thromboplastin, and then was weak in its action. Barium was incapable of producing coagulation even in the presence of thromboplastin. It was found, in this connection, that the presence of thromboplastin allowed coagulation at lower levels of calcium and strontium than when it was absent, and the more thromboplastin, the lower the requisite concentration of the calcium or strontium ion. Changes in calcium level in physiologic and pathologic states are never within the extreme ranges of alteration in the experiments, and are probably never sufficient to alter the speed of coagulation in the human patient. SE

HEMORRHAGIC DIATHESIS IN HIROSHIMA, NAGASAKI AND AT BIKINI ATOMIC BOMB TEST. A. L. Copley. From the Laboratory of Cellular Physiology, Department of Biology, New York University, N. Y. C. J. Nat. Med. 137: 145, 1948.

It is known that petechial hemorrhages and thrombocytopenia occurred after the atomic bomb explosions, and that, in addition to thrombocytopenia, hyperheparinemia occurs after exposure of dogs to x-rays, and is in part responsible for the resultant bleeding tendency. The present author seeks to correlate the occurrence of hyperheparinemia and thrombocytopenia, with resultant hemorrhage, by pointing out that (1) heparin induces clumping of platelets, thereby producing platelet-agglutination *in vitro*, and thrombocytopenia *in vivo*, and (2) the injection of heparin in various animals induces not only petechiae but also white emboli (platelet-clump plus white blood cells), which also result in vascular damage. He suggests, therefore, that ionizing radiation may produce its hemorrhagic effect by inducing hyperheparinemia (mechanism unknown), the heparin then clumping platelets (thrombocytopenia plus petechiae) and causing white embolization (petechiae).

The observations upon which these suggestions are based—i.e., the effect of heparin on platelets, etc.—are as yet unconfirmed. SE

## LEUKEMIA AND LYMPHOMA

EFFECTS OF RADIOACTIVE SODIUM ON LEUKEMIA AND ALLIED DISEASES. T. C. Evans, M. Lenz, C. P. Driscoll, and M. J. LeMay. Department of Radiology, Columbia University, N. Y. C. Am. J. Roentgenol. 54: 469-481, 1948.

$\text{Na}^{24}$  (half-life 14.8 hours) was given orally in single doses of about 20 millicuries. The amount of radiosodium in urine was less than 10 per cent of administered dose. Cases treated included chronic myelogenous leukemia (7), subacute and acute leukemias (4), chronic lymphatic leukemia (4), acute lymphatic leukemia (2), polycythemia vera (1), sympatheticoblastoma (1). Case reports are presented for many of the patients. There was no irradiation sickness. In chronic leukemia changes in the peripheral blood were similar to those following other types of irradiation. However, the therapeutic results were not superior to those with  $\text{P}^{32}$  or x-ray, and the short half life of the isotope would make its general use impractical. C. F.

DISCUSSION ON CHEMOTHERAPY IN MALIGNANT DISEASE *A Haddow, E Paterson, I A Thomas, E W Rubes, E Boyland* Proc Roy Soc Med 41 45-51, 1948

This is a largely clinical discussion on recent attempts to influence malignant disorders by various chemical means. Included are short discourses on estrogens in carcinoma of the prostate, chloroethylamines (nitrogen mustards) in Hodgkin's disease, and urethane in leukemias.

S E

NITROGEN MUSTARDS IN THE TREATMENT OF HODGKIN'S DISEASE AND LYMPHOSARCOMA *M Sherry* From Sinai Hospital, Baltimore South M J 41 118-129, 1948

Experiences are reported with the use of nitrogen mustard in the treatment of a small series of cases of lymphomatous disorders. The results duplicate those found in other centers: there was no effect in two patients with melanocarcinoma and two patients with lymphosarcoma, a fair response in a patient with giant follicle lymphosarcoma, and excellent responses in five of six patients with Hodgkin's disease. Relapse in the latter cases occurred within four months in most instances, although one patient was still in remission 11 months after treatment. It was noted that the lymphocytes in the blood typically became pale, granulated, deformed, and otherwise changed after nitrogen mustard, showing changes of noxious nature.

S E

THE TREATMENT OF BOECK'S SARCOID WITH NITROGEN MUSTARD: A PRELIMINARY REPORT *G E Snider* From the Veterans Administration Hospital, Fort Howard, Maryland South Med J 41 11-14, 1948

This short clinical report, admittedly preliminary and inconclusive, seeks to suggest that the use of nitrogen mustards may accelerate remissions in widespread sarcoidosis. Four cases are presented, in all of whom the diagnosis was established ultimately by node biopsy, and in all of whom there was involvement of eyes, lungs, nodes, and sometimes skin. Methyl-bis (beta chloroethyl) amine hydrochloride was administered in two courses one month apart, and the patients followed for signs of change. Marked retrogression of enlarged nodes, lung infiltrations, and ocular and other abnormalities occurred in the first three cases, beginning from two weeks to three months after initiation of treatment; the fourth case remained stationary.

The natural course of the disease makes evaluation of this and any similar short-term report most difficult, as the author himself points out. A complicating factor is the presence of tuberculosis in case 2, as demonstrated by sputum inoculation of a guinea pig, and it is at best questionable whether nitrogen mustards should be used in tuberculosis. It is true that many of the changes to normal were remarkable, but whether chloroethyl amine played a role in these changes cannot be deduced from the evidence presented.

S E

BOECK'S SARCOID IN CHILDREN *R J Reeves, G J Baylin, and P A Jones* From the Department of Radiology, Duke Hospital and Duke University School of Medicine South Med J 41 295-302, 1948

This is a review of thirteen children with sarcoidosis, aged from 9 to 14 years, in whom observation was made for periods as long as seven years. The authors point out that there are only twenty-one previously reported cases in the literature, and that the disease, although uncommon, is not rare in the young age groups.

S E

## HEMATOPOIETIC TISSUES

ISOLATED CHROMOSOMES *A E Mirsky and H Ris* From the Laboratories of the Rockefeller Institute for Medical Research, New York City J Gen Physiol 31 1-6, 1947

Erythrocytes of the salmon carp and fowl have been used as a source for obtaining isolated chromosomes. The procedure for the isolation of chromosomes was the first part of a method used by the authors to prepare a desoxyribose nucleoprotein complex-chromosin. The isolated threads showed a definite organization along their axis: a tightly coiled helix and at least two chromonemata. Since few of the

cells were in mitosis, the isolated chromosomes were liberated from interphase nuclei. This is additional evidence that chromosomes retain their individuality during interphase, or the so-called resting stage.  
O P J

THE CHEMICAL COMPOSITION OF ISOLATED CHROMOSOMES *A E Mirsky and H Ris* From Laboratories of the Rockefeller Institute for Medical Research, New York City *J Gen Physiol* 31 7-18, 1947

Lymphocytes of the calf thymus are a convenient source for obtaining isolated chromosomes. The method of isolation was somewhat similar to that used for fish and fowl blood. By fractionation of the isolated chromosomes it was shown that 90 to 92 per cent of the mass was largely desoxyribose nucleohistone. The insoluble residue (or residual chromosome) contained a nonhistone, tryptophane-containing protein, 12 to 14 per cent ribose nucleic acid, and about 2 per cent desoxyribose nucleic acid. For years hematologists and cytologists have described basichromatin and oxychromatin. It now appears that these two chromatin differ chemically.  
O P J

EFFECTS OF EXPERIMENTAL CRYPTORCHIDISM UPON THE THYMUS, MEDIASTINAL LYMPH NODES AND ACCESSORY SEX GLANDS OF THE ALBINO RAT *J C Plagge* From the Department of Anatomy, University of Illinois College of Medicine, Chicago, Illinois *Anat Rec* 98 597-608, 1947

The use of cryptorchid animals made it possible to study the influence of the secretion of the interstitial tissue of the testis upon the thymus and mediastinal lymph nodes. Unlike the results obtained in castrated albino rats, there was no striking enlargement of these organs.  
O P J

THE CYTOPLASMIC BASOPHILIA OF BONE-MARROW CELLS *J C White* From the Department of Pathology, The British Postgraduate Medical School, Hammersmith, London *J Path and Bact* 59 223-234, 1947

There has been an increasing amount of evidence to substantiate the concept that cytoplasmic basophilia and a high ribonucleic acid content are characteristic of young and actively growing cells. White has contributed to this by applying Brachet's cytochemical test to bone marrow aspirations from six normal individuals and nineteen with various pathologic conditions. Ribonuclease abolished the basophilia of the cytoplasm and nucleoli in proerythroblasts and myeloblasts. In such cells a fine ring of nucleolus associated chromatin remained which was more deeply staining in the former. After nucleoli have disappeared in the more mature cells, distinct and deeply staining chromatin persists at their site. In pernicious anemia, hemocytoblasts, young normoblasts and young megaloblasts had a high content of cytoplasmic ribonucleic acid. The granular nature of basophilic cytoplasm was shown by the use of acid fuchsin to be due to the presence of numerous mitochondria.  
O P J

FURTHER OBSERVATIONS ON THE CHEMICAL CYTOLOGY OF MEGAKARYOCYTES AND OTHER CELLS OF HEMOPOIETIC TISSUES *G B Wislocki, H Bunting and E W Dempsey* From the Department of Anatomy, Harvard Medical School, Boston, Mass *Anat Rec* 98 527-538, 1947

Bone marrows from several rhesus monkeys and six guinea pigs were studied after applying various cytologic and histochemical techniques. Both the basophilia and metachromasia of megakaryocytic cytoplasm were abolished after digestion in a solution of ribonuclease. Although this was true for the rhesus monkey, it was not entirely so for guinea pig megakaryocytes, because they retained some metachromasia after exposure to the enzyme. The distribution of mitochondria seemed to be identical with that of the cytoplasmic objects with an affinity for sudan black. Blood platelets exhibited a faint basophilia and metachromasia. The presence in blood platelets of lipidal particles, mitochondria and bodies mixed supravitaly with neutral red similar to those found in the cytoplasm of megakaryocytes reaffirmed Wright's theory for their origin. Further studies were also made on tissue eosinophils and basophils.  
O P J

METACHROMASIA IN MAMMALIAN TISSUES AND ITS RELATIONSHIP TO MUCOPOLYSACCHARIDES *G B Wislocki, H Bunting, and E W Dempsey* From the Department of Anatomy, Harvard Medical School, Boston, Massachusetts *Am J Anat* 81 1-38, 1947

Metachromasia is of interest to the hematologist because certain blood cells possess that property of inducing chemical and chromatic changes when stained with suitable dyes. Wislocki and his associates have investigated this in tissue sections of material from thrombocytes fixed in four per cent basic lead acetate and stained with one half per cent toluidin blue. Some metachromasia is due to the presence of mucopolysaccharides, but in other instances it may be referable to the presence of other nucleoproteins and substances of unknown composition. Tissue mast cell granules have a strong affinity for both toluidin and methylene blue. The metachromatic reaction was unaltered by exposure to hyaluronidase. These granules did not give the Baue reaction after digestion with saliva. The authors were unable to abolish the metachromasia present in metachromatic cytoplasm after exposure to ribonuclease.

O P J

AN IMPROVED METHOD OF STAINING LEUCOCYTES WITH SUPRA-BLACK. *B. H. I. Stetson and G. B. Storer*. From the Pathology Department, University of Liverpool. J. Path. and Bact. 53: 336-337, 1947.

The authors have developed a new method for staining with supra-black in differentiating various granules as seen in dry smears of normal blood. The results have been very consistent and better than those obtained previously. Such things as fixation in formaldehyde vapor and the use of a buffered solution containing phenol have improved the technique. Sheehan and Storer attribute a mordanting action to the phenol. In this connection it must be pointed out that another interpretation of the action of phenol is possible, namely, that of lipophanerosis. The authors made the interesting comment that in certain blood diseases neutrophilic (polymorph) leukocytes do not contain cyanophilic granules. This will be described in a subsequent paper.

O P J

THE EFFECTS OF LEAD SALTS ON THE HEMATOPOIETIC AND HISTIOCYTIC SYSTEMS OF THE LARVAL FROG. *H. C. Barrett, Jr.* From the Department of Anatomy, Western Reserve University, Cleveland, Ohio. Am. J. Anat. 81: 117-138, 1947.

The effects of solutions containing 1 part lead salt to 40,000 parts water on the hematopoietic systems of larval bullfrogs was studied in 40 control and 143 test animals. Although both lead acetate and lead nitrate produced similar results, the action of the latter was more rapid. Among the first changes observed were those in the mature erythrocyte which became fragile and subject to fragmentation. There was a subsequent increase of immature erythrocytes and hemoblasts. Differentiation seemed to occur in the peripheral blood with the eventual formation of some spheroidal erythroplastids. The liver, which is not normally a hematopoietic organ in the tadpole, developed lymphogranulopoietic areas in the perportal connective tissue. In advanced stages of lead poisoning degenerating erythrocytes were present in the sinuses of the spleen. In this situation they remained unphagocytized because of toxic alterations in the histiocytes. Normally the intertubular mesonephric connective tissue of the larval frog is the chief hematopoietic center. This became rapidly depleted of its cellular elements and, for some unknown reason, eosinophilic myelocytes actually increased there.

O P J

INTERCELLULAR CEMENT AND CAPILLARY PERMEABILITY. *R. Chambers and B. W. Zuerbach*. From the Department of Biology, Washington Square College of Arts and Sciences, New York University, New York. City Physiol. Rev. 27: 436-463, 1947.

This review of the basic anatomic physiology of cellular membranes—specifically the capillary wall—discusses fundamental rather than clinical interpretations of the problems of capillary tonus, permeability, and fragility. From the point of view of structure, three types of membrane may be discriminated: (1) one in which the cell itself determines the permeability of the membrane (e.g., the proximal tubule of the kidney), (2) one in which both the cell and the intercellular material determine the permeability (e.g., intestinal mucosa), and (3) one in which permeability of the membrane is determined exclusively or largely by the intercellular material. Into this last group the authors place the capillary membrane.

The capillary wall consists of endothelial cells plus an interendothelial cement elaborated by these cells, an endocapillary lining which seems to be an adsorbed layer of a blood protein, and a pericapillary sheath of connective tissue. With regard to the interchange of material between blood and tissue (i.e., permeability of the capillary), the interendothelial cement substance serves as the basic framework. It acts as an ultrafilter the selective properties of which depend directly on variations in its porosity.



Its porosity (and therefore the permeability of the membrane) is high, as compared with the permeability of the endothelial cells themselves, which is low. The porosity of the intercellular material may be affected by mechanical stretching, by chemical and pH changes of the surrounding medium, etc. This cement substance is thought to be produced by the endothelial cells, and to be in a constant state of usage and replacement.

The endocapillary lining seems to be an adsorbed layer of blood protein, probably albumin, which penetrates the porous interstices of the cement and helps reduce the pore size of the cement filter. In this way, it helps to reduce capillary permeability. The pericapillary sheath is a condensation of connective tissue which serves mechanically to support the blood capillary.

Changes may occur in any of the three components of the capillary and result in increased permeability. It is of interest that the variations in capillary permeability can be explained by changes in the nonliving components of the capillary wall: the intercellular cement, the endocapillary protein, and the pericapillary connective tissue sheath. The endothelial cell itself apparently often merely produces the cement substance. It is apparent, correspondingly, that it may be difficult to explain a given change in permeability on a single basis, and that it is unreasonable to assume a single permeability factor to account for fluid balance in the body.

This article discusses further the topography of the capillary bed, the roles of precapillary vasomotion and of hydrostatic and colloid osmotic pressures in permeability, the nervous control of capillary permeability, and the significance of various agents affecting capillary permeability. The manner of action of histamine, adrenal cortex, and various agents affecting capillary permeability, are discussed.

S E

THE EFFECTS ON VENOUS ENDOTHELIUM OF ALTERATIONS IN BLOOD FLOW THROUGH THE VESSELS IN VEIN WALLS AND THE POSSIBLE RELATION TO THROMBOSIS. *J. F. O'Neill*. From the Department of Physiology, Harvard School of Public Health, Boston, Massachusetts. *Ann Surg* 126: 270-288, 1947.

An ingenious technic is presented for mounting flat segments of vein walls so that the inner coats of the wall can be stained and studied. Benzidine, which selectively stains red blood cells, was used to outline the vascular plexus in vein walls, and silver nitrate was employed in staining and studying the inner coats of vein walls.

Increasing endothelial desquamation was found in vein segments which were dissected free from all blood, lymphatic and nerve supply to the walls, but which were left unobstructed *in situ*. Despite the presence of raw, muscular surfaces, however, intravascular clotting seldom occurred, presumably because the velocity of blood flow was maintained. When blood flow was reduced by eighty to ninety per cent following the application of small metal clamps without isolating the vein from its bed, there was less resultant endothelial damage but slightly greater tendency toward thrombosis. When isolation and partial obstruction of vein segments were combined, there was a high incidence of intravascular clotting but it is acknowledged that further experience with this procedure will be required before firm conclusions can be drawn regarding the mechanisms involved.

I E Y

CULTURE OF BONE MARROW *IN VITRO*. *A. Freschi and G. Astaldi*. Pavia, Italy, Tip. del Libro, 1946.

The Italian authors have assembled in a book the results of twelve years experience concerning bone marrow.

They studied in succession normal marrow, pernicious anemia, leukemia, and erythroblastosis.

*In vitro*, normal marrow loses its organic characteristics, immature granulocytes develop in a slow and restricted manner. During the course of this development, one may see a new generation of granuloblastic cells which are derived either directly from hemocytoblasts or possibly from pre-existing granuloblasts. Erythroblasts develop much more rapidly and completely than granulocytes to the orthochromatic stage or even after a reticular formation, arrive at the red anuclear globular stage. Very few immature erythroblasts develop to replace the elements which have evolved.

When after fifteen to twenty days the parenchymal elements have gradually disappeared the histiocytes develop and are soon transformed into a pure culture of fibroblastic elements.

In myeloid leukemia, one meets a similar evolution; the degree of development is no different; a different time of survival of the parenchymal cells is analogous.

In lymphoid leukemia the same time of survival and development of the fibroblastic cells is observed. One may note, in addition, morphologic modifications of the lymphocyte—the chromatin less thick, the protoplasm more diffused—and this would not be a matter of actual return to the most immature forms but the mere adaptation of the cells to a new environment. It is important to note that the histiomonocytic cells observed do not ever result in lymphocytic elements; the matter concerns two separate developments, and the differences can only make themselves plain successively in the culture: the lymphocyte disappears when the histiocytes are transformed into the fibroblasts, which alone survive.

In acute leukemia, a new fact is obtained: the possible survival of parenchymal elements after a month of culture. Different in structure, the root cell of acute leukemia do not give rise to macrophages or to true monocytes, nor to fibroblasts. The extremely polymorphic elements which can take shape, although of histoid morphology, are of hemato-myeloblastic appearance but do not appear to form any cells of a normal grouping. The cells of acute leukemia are of the "preparenchymal" type which doubtless explains their particular faculty for survival, though they do not evolve toward the fibroblastic state.

Thus chronic leukemia and acute leukemia appear to possess very different histogenesis.

The authors expect shortly to issue a classification of the leukemia. We limit ourselves here to a brief resume of the results of medullary culture in the leukemias which displays at the same time the interesting aspects and the actual limits of the method of study to which Hirsch and Astaldi have devoted themselves.

J P S

THE EFFECTS OF EXPERIMENTAL HYPERTHERMIA AND RESTRAINT ON THE BLOOD AND HEMATOPOIETIC ORGANS OF THE ALBINO RAT. J. C. Latta and W. P. Nelson. From the Department of Anatomy, College of Medicine, University of Nebraska, Omaha, Nebraska. *Am J Anat* 6: 321-351, 1945.

The authors studied the effects of sustained fever on the blood values of white rats, and in a preliminary study they used as controls two animals which were untreated except for handling and one which was restrained but untreated. Quite unexpectedly the restrained animal showed marked changes. Hence the problem of determining what effects on the blood and blood-forming organs were attributable to hyperpyrexia alone arose. Albino rats were placed in a Kettering hypertherm and after rectal temperatures reached  $103-104^{\circ}\text{F}$ , they were maintained at this temperature for five hours. Blood samples were obtained from the tail. Both sustained fever and restraint produced a marked lymphopenia after the first hour. Neutrophils first increased then decreased moderately and finally increased again during the fourth and fifth hours of hyperpyrexia. In the case of restraint, neutrophils increased steadily to twice the absolute value in five to six hours. This increase was not sufficient to offset the lymphopenia in heat-treated animals. Consequently, a leukocytosis did not occur. Tissue studies indicated a relative marrow depression in hyperpyrexia and no evidence of a neutrophilic infiltration in various organs. The marked lymphopenia was accounted for in part by a failure of delivery and to a greater extent by degeneration and destruction of lymphocytes.

O P J

HEMATOPOIESIS IN THE EUROPEAN PLETHODONTID, *HYDROMANTES ITALICUS*, WITH REFERENCE TO PHYLOGENY. W. C. Barrett, Jr., From the Department of Anatomy, Western Reserve University, Cleveland. *Anat Rec* 98: 127-136, 1947.

Most hematologists are so engrossed with problems related to clinical hematology that not infrequently they forget about the vast and relatively unexplored field of comparative hematology. In the human, some disturbances of the hematopoietic organs may be recognized and classified according to the type, amount and distribution of hematopoietic tissue. Biologists have put somewhat similar information to an entirely different use. Knowledge that hematopoietic loci may be variously distributed in the organs of amphibia, and that they may be either lymphocythropoietic or lymphogranulopoietic, has made it possible to determine the phylogenetic position of certain genera.

O P J

SHRINKAGE OF LYMPHATIC TISSUE IN RATS FOLLOWING INJECTIONS OF INSULIN. I. T. Zecker. From the Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania. *Am J Physiol* 152: 267-270, 1948.

In view of the work of Selye with the alarm reaction, and of White and Dougherty with an interrelationship between adrenal cortex and lymph nodes, it was thought of value to see whether other agents known to affect the adrenal glands might lead to similar effects in the lymph nodes. Specifically, since insulin is known to stimulate the adrenal medulla by way of the sympathetic nervous system, would the same results upon lymphoid tissue occur following this endogenous release of epinephrine, as occur, for example, following an alarm reaction, or the injection of (exogenous) adrenalin?

The author injected one group of rats with epinephrine daily for three to seven days, and another group of rats with ascending doses of insulin daily for three to seven days. Other rats served as controls. The rats were then killed and their tissues weighed. With few exceptions, it was found that adrenal tissue enlarged after insulin or epinephrine, and that lymphatic tissue shrank after insulin or epinephrine.

The author postulates that various stimuli (e.g., injury, insulin) which cause discharge of adrenal medulla secretion, in so doing also cause slower but more sustained cortical activity (after injury, for example, the cortex enlarges, loses its lipids, and secretes its hormones). Hence, perhaps, the adrenal medulla is a link in the pituitary-cortex interrelationship, and, also, stimuli which affect this relationship may therefrom affect the lymph nodes.

Although no blood counts were done, mention is made of a previous observation that insulin produces lymphocytopenia and dissolution of cells in lymph nodes (Latt, J. S., and Henderson, J. W., *Folia haematologica* 57: 206, 1937), i.e., a reaction similar to that found by Selye following an alarm reaction, and by White and Dougherty following the injection of adrenal cortex.

S E

AN EXPERIMENTAL STUDY OF LYMPH NODE REGENERATION IN RABBITS *W. J. Furuta* From the Department of Anatomy, University of Illinois College of Medicine, Chicago, Illinois. *Am. J. Anat.* 80: 437-503, 1947

Although many reports have been made on the regeneration of lymph nodes, they have not been too careful in reporting the age of the animal, method of node removal and length of postoperative period. The results in the present article were obtained after a study of 270 specimens of popliteal nodes obtained by unilateral and bilateral excisions of 152 rabbits (three days to three and one-half years in age). Popliteal lymph nodes prior to fixation measured  $8 \times 12$  mm. Supernumerary nodes were very rare. Regeneration, progressive differentiation of undifferentiated tissue, was optimum in incidence and quality in rabbits about one and one-half months old, thirty days postoperatively. This occurred from lymphoreticular tissue in perinodal areolar tissue. One of the most outstanding findings was that senile nodes may be activated by typhoid vaccine so that there is perhaps no complete loss of structure and function.

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AGE CHANGES IN LYMPH NODES *F. A. Dineen* From the Department of Morbid Anatomy, University College Hospital, Medical School, London. *J. Path. and Bact.* 59: 575-591, 1947

Over three hundred lymph nodes from deep cervical, inguinal, bronchial, mesenteric and axillary groups were obtained from 150 autopsies. Three groups of subjects were equally divided among those derived from (a) accident cases, (b) acute medical and surgical cases, and (c) chronic disease cases. With respect to structural differences and response to aging, lymph nodes may be divided into two groups—superficial and deep. There is a difference between cortical and medullary reticulum (argyrophil fibres) in both. In the cortex the meshwork is larger, more open and polyhedral in shape than in the medulla. Superficial nodes undergo retrogression at puberty. This is characterised by a shrinkage of lymphoid tissue on the capsule rather than the hilum which results in a cup-like structure filled with connective tissue. Germinal centers of the superficial nodes are fewer in number, smaller in size and they possess smaller pale centers than those of deep nodes. Although there is a diminution in size of germinal centers of deep lymph nodes from puberty onward, they can still be found at the age of eighty. Retrogressive changes in deep lymph nodes are unlike those in the superficial group in that there is a gradual return to the fetal type of node.

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contraction and emptying of the postulated stored blood in human adults. He injected blood containing labeled red blood cells (labeled with radioactive phosphorus according to a technic of the author) into 5 healthy men, and took blood samples in 10 and again in 15 minutes after the injection. The subject was then made to do severe muscular work, and two further blood samples taken, the first at 25-30 minutes, the second at 27-39 minutes. All samples were subjected to radioactivity determinations in a Geiger-Müller counter. It had previously been shown by the author that the radioactivity of the blood remains constant for at least 60 minutes after such an injection, hence, the volume of the circulating red cells could be measured by the radioactivity of the blood. Presumably, if any reservoir of blood was present which responded to the severe muscular exercise, discharge of red cells from this reservoir would change the radioactivity of the circulating blood.

Nylin found, actually, that there was no significant change in the specific radioactivity of the blood after exercise within the time studied. For all the patients, the mean circulating cell volume before work was 2,408 ml, as compared with 2,471 ml after work, and the mean circulating total blood volume before work was 4,934 ml, as compared with 4,855 ml after work. Since the amount of the red cells was unchanged, it was concluded that there was no reservoir which empties red cells into the circulation after work. This result is in contrast with the work of Bancroft (in dogs), and with the commonly held opinions that epinephrine contracts the spleen and thereby increases the numbers of circulating red cells. If verified, these conclusions would be of great theoretic importance.

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# BLOOD

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### HISTORICAL NOTES ON BLOOD PLATELETS

By LEANDRO M. TOCANTINS, M.D.

ON MARCH 7, 1842, at a session of the "Académie des Sciences" of Paris during an account of studies on the microscopy of blood, Donné<sup>1</sup> stated in clear terms that there existed in the blood, red and white globules and little globules ("globulins"). There seems to be no earlier record of the recognition of the platelet as a formed element of the blood. Later, in his book "Cours de Microscopie," Donné<sup>2</sup> repeated and amplified his original description. Although his interpretation of the origin of the "globulins" (the lymph) was erroneous, the substance and manner of his opening statement indicates that he had clearly recognized the existence of a distinct morphologic element (fig. 1). Signs of awareness of the presence in the blood of forms other than white and red cells were evident before Donné. In Hewson's and Andral's<sup>3</sup> works, references are made to certain white globules and bodies distinct from leukocytes and red corpuscles but of uncertain nature, occurrence and identity.

At about the same time as Donné, Zimmermann described certain bodies which he believed were the precursors of red blood cells. He called them "Elementarbläschen" and remarked on their tendency to gather in clumps. Zimmermann<sup>4</sup> appears to have been among the first to use anticoagulants in the cytologic study of blood. He bled a horse and collected the blood in a solution of 6 per cent magnesium sulphate, equal parts of blood and solution. In this mixture he found what were undoubtedly platelets.

Among the first attempts to attribute the origin of platelets to other blood elements is the work of Schultze<sup>5</sup> (1865). He had observed in normal blood small elements which he considered to be of protoplasmic nature. These had a strong tendency to clump and form granular masses ("Kugel") which he did not consider identical with Zimmermann's "elementar blaschen"; he thought they resulted from the destruction of white corpuscles. Schultze's hypothesis was corroborated in 1872 and subsequent years by Riess,<sup>6</sup> who maintained that during anemias and cachectic states, the white corpuscles fragment and give rise to smaller corpuscles which he called "zerfallskörperchen" (disintegration bodies), analogous to the "Kugel" of Schultze, but differing from Zimmermann's "Elementarblaschen".

Throughout the third quarter of the past century one finds, here and there, what were probably platelets described as bacteria.<sup>7</sup> The morphologic variability of

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platelets, depending on the conditions of collection and observation of the blood, was perhaps the reason for regarding them as extrinsic matter, peculiar to certain pathologic conditions. In 1873, Vulpian<sup>8</sup> noted the presence in the blood of colorless corpuscles having the properties of sticking to the cover glass and accumulating in clumps. In the same year, Ranvier<sup>9</sup> observed in the center of the fibrinous network that appears during coagulation of blood, granulations with tinctorial characteristics different from those of leukocytes and erythrocytes. He advanced the

PHYSIOLOGIE — *De l'origine des globules du sang, de leur mode de formation et de leur fin*, par M. AL. DONNÉ (Extrait par l'auteur)

(Commissaires, MM. Magendie, Flourens, Dumas, Milne Edwards, Payen)

« Il existe dans le sang trois espèces de particules : 1° les globules rouges ou sanguins proprement dits, 2° les globules blancs qui n'ont été bien connus que dans ces derniers temps, 3° les globulins du chyle.

• Les globules rouges sont plats dans toutes les espèces de sang; ils sont circulaires dans le sang des mammifères, et elliptiques dans celui des oiseaux, des poissons et des reptiles.

FIG. 1. EXCERPT FROM DONNÉ'S REPORT BEFORE THE PARIS ACADEMY OF SCIENCES (1842)

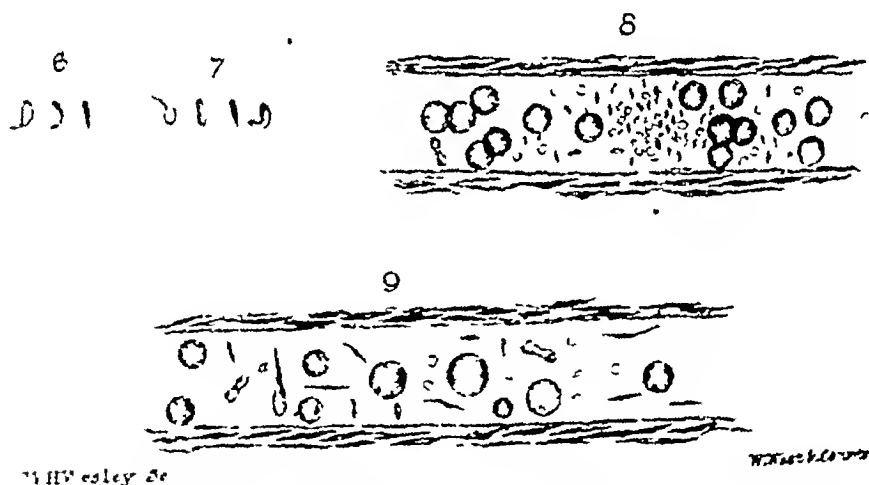


FIG. 2. DRAWINGS BY WILLIAM OSLER ILLUSTRATING THE ORGANISMS HE OBSERVED IN THE VENULES OF FRESHLY KILLED RATS (1874)

view that these masses probably determine the coagulation of blood, very much in the same way as the crystal of a salt brings about the crystallization of a saturated solution of that salt.

In 1874, Osler<sup>10</sup> pointed out that the "granular masses" of Schultze result from the agglutination of small bodies which occurred as single units in the circulation. He demonstrated, and his illustrations clearly showed for the first time, that these granular masses occurred as single elements in the circulating blood (fig. 2), and came together when the blood was shed. Osler's observations were carried out in the venules of recently killed young rats, and mark the time when the platelet be-

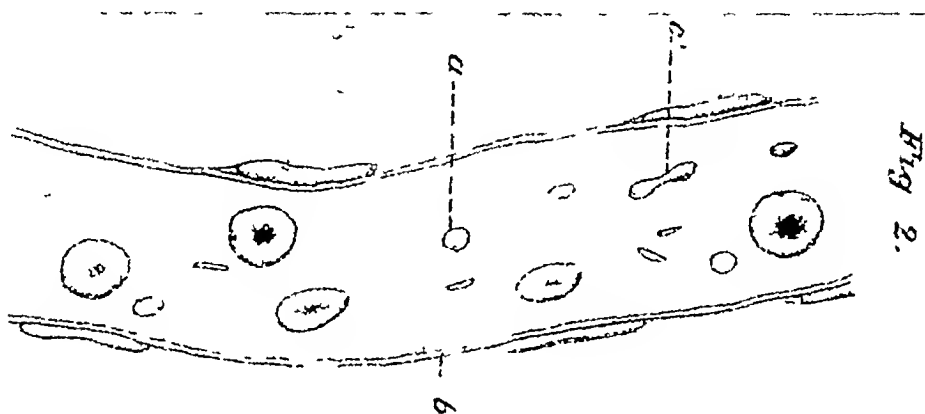
gan to be held, not as an artefact or a by-product of a change in shed blood, but as a normal constituent of the circulating blood itself

A few years later, Hayem<sup>11</sup> pointed out that "il existe dans le sang de tous les vertébrés des petits éléments qui ne sont ni des hématies, ni des globules blancs". He, however, believed that the elements represented primitive red corpuscles that had not gone beyond a certain phase in their evolution, for this reason he called them "hematoblasts". Hayem attributed to the hematoblast a double function: it was a coagulation accelerating agent ("le sang porte dans son sein un hémostatique puissant")<sup>12</sup> and it played a role in the regeneration of blood, a conclusion based on his observations on changes in the platelets after hemorrhage and in acute diseases. Some of Hayem's first studies were concerned with the evolution of the red blood cells in the blood of oviparous and viviparous vertebrates. It was these and subsequent studies that led him to consider the platelet as a precursor of the red blood cell. Without the breadth of his training and experience it was, and is, difficult to follow his views. Today, the available evidence makes it unlikely that such a relationship between platelets and red blood cells exists, as claimed by Hayem. The prevailing view of the origin of platelets renders it improbable that a body that arises from fragmentation of the cytoplasm of megakaryocytes may later be transformed into a red blood cell. Moreover, the morphologic features of the precursors of red blood cells are now well established and differ substantially from the platelet.

The existence of the platelet as a distinct element in blood within the vessels was further confirmed by Bizzozero<sup>13, 14</sup>. He seems to have been the first to observe the platelet circulating in the blood of living animals. He stressed that the "granular masses" of Schultze were neither residues of white corpuscles which were destroyed before or after collection of the blood, nor granulations of fibrin, as thought by Ranvier. The masses were derived from special morphologic elements pre-existing in the blood, which he called "plättchen". Bizzozero was probably influenced by the expression "blaschen" (globule, vesicle) that had been previously used by Zimmerman. It was Bizzozero<sup>13</sup> who established securely the foundation for the present day conception of the platelet as a distinct element of the circulating blood, ("Einen neuen Formbestandteil des Blutes") (1882), and indicated the part it plays in thrombosis. Before his work, the white thrombi were considered to be made up principally of leukocytes<sup>15</sup>. By a number of ingenious experiments, Bizzozero demonstrated that the white portion of these thrombi consisted almost exclusively of platelets, gradually accumulated at a point in the vessel where the wall had been injured or the circulation obstructed. Once accumulated in a mass, "in vivo" or "in vitro," the platelets underwent changes in appearance, became unusually sticky, a phenomenon he designated as "viscous metamorphosis". Bizzozero's observations had the merit of having been made on the circulating blood of a living animal (fig. 3). Osler's observations were made on the vessels of dead animals, it was perhaps because of this fact that, at the time of his report, Osler<sup>7</sup> was not quite clear in his mind whether these bodies might be bacteria. The title of his paper "An Account of Certain Organisms Occurring in Liquor Sanguinis"<sup>10</sup> carried that implication. The expression "plaque" was not used by Bizzozero<sup>16</sup> in his



work until 1891, and seems to have been introduced by Hayem<sup>17</sup> in 1883. Bizzozero originally referred to these bodies as 'petites plaques' or simply 'plaques' (fig 3). The English word 'platelet' does not seem to have been used until later in the 19th century. Osler<sup>18</sup> was among the first to translate Bizzozero's expression 'Blut Plattchen' as 'Blood Plates' although he did not consider it a good descriptive word. Other expressions used to designate these elements were 'blood plaques,



En examinant avec un objectif à immersion le contenu de ces vaisseaux (veines ou capillaires) on arrive à ce résultat surprenant, qu'en réalité à côté des globules rouges et des globules blancs circule un troisième élément morphologique (fig 2). Il est représenté par de petites plaques ← très pâles de la forme de disques à surfaces parallèles ou, plus rarement, de lentilles ovales ou rondes, d'un diamètre égal au tiers ou à la moitié de celui des globules rouges. Ces plaques sont toujours incolores et circulent dispersées irrégulièrement entre les autres globules, ne montrant point de préférence pour la partie centrale plutôt que pour la partie périphérique du courant. Ordinairement elles sont isolées les unes des autres, ce qui n'empêche pas cependant que souvent on ne les trouve réunies en groupes plus ou moins grands. Cette agglomération est déjà

FIG 3 ILLUSTRATION AND TEXT IN BIZZOZERO'S PAPER, DEMONSTRATING THE EXISTENCE OF PETITES PLAQUES IN BLOOD WITHIN THE VESSELS OF LIVING ANIMALS (1882)

'disklets,'<sup>19</sup> 'third corpuscles,'<sup>20</sup> 'fugitive corpuscles,' 'fugitive discs,' 'invisible colourless discs'<sup>22, 23</sup>

Among those who lent their support to the discovery of the new morphologic element was William H. Howell who died in February 1945, after a career in physiology extending over a half a century. His first paper corroborating and extending Bizzozero's observations was published in 'Science' in 1884.<sup>24</sup> One of his last papers proposing the lungs instead of the bone marrow as the principal source of platelets appeared in 1937.<sup>25</sup>

Bizzozero's monograph gave rise to much opposition from the pupils of A. Schmidt<sup>26</sup> and Lowit<sup>27</sup> who adhered to the theory that the bodies designated as

"Plättchen" by Bizzozero were derived from fragmentation of leukocytes. Much of the disagreement and contradiction in papers that followed Bizzozero's may be attributed to technical difficulties that, even today, try the patience of those engaged in the study of platelets. The many shapes and arrangements presented by these bodies have confused some investigators, whose opinions rested mostly on morphologic evidence obtained "in vitro." Bizzozero also departed sharply from

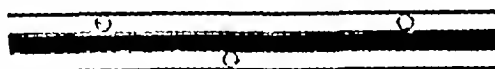


Fig 1 Normaler, schneller Blutstrom, axialer Character

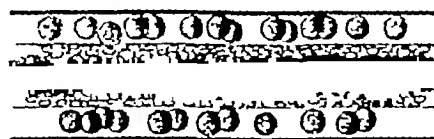


Fig 2 Randstellung der Leucocyten, geringe Verlangsamung der Circulationsgeschwindigkeit.

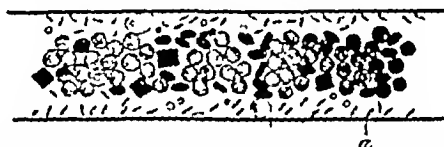


Fig 3 Blutplättchen a in der plasmatischen Randzone. Starke Verlangsamung der Circulationsgeschwindigkeit. Abnahme der Randstellung der Leucocyten.

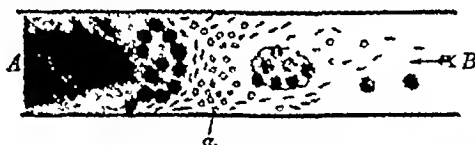


Fig 4 Stagnation. Nach A bräunliche Thrombose, nach B blaue Communication mit einem strömenden Gefässe. Plättchen a zum Theil verändert (zackig).

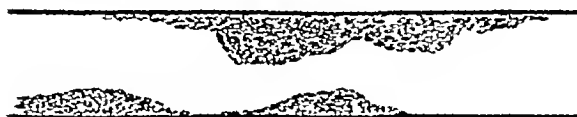


Fig 5 Wandständiger Blutplättchentrombus

FIG 4 ILLUSTRATIONS OF THE PAPER BY EBERTH AND SCHIMMELBUSCH DEPICTING THE MODE OF FORMATION OF PLATELET THROMBI (1885)

the view held by Schmidt<sup>26</sup> that the fibrin ferment originated from the destruction of leukocytes. Among the opponents of Bizzozero's ideas were Weigert,<sup>28</sup> who attributed the findings of the Italian investigator to artefacts resulting from vessel compression, circulatory disturbances and the anesthesia employed. Bizzozero countered these objections by repeating and confirming his own observations on the intact vessels of the wing of a living unanesthetized bat.<sup>16</sup>

Wooldridge<sup>29</sup> on the basis of experiments with incoagulable bloods, especially after the injection of peptone, believed that platelets were simply a precipitate of

the globulin portion of the plasma ( 'Globulin Plattchen ' ) and not a distinct element of the blood Lowit<sup>27</sup> supported Wooldridge's contention and brought out much evidence against the existence of the platelet The idea that the platelet, as such, does not exist in the blood is still encountered in modern texts,<sup>30</sup> a repercussion of the observations of Wooldridge The observations of Bizzozzer<sup>31</sup> were later confirmed and extended by Eberth and Schimmelbusch<sup>31</sup> and Laker<sup>3</sup> while studying the circulating blood in the vessels of living dogs and other mammals Eberth and Schimmelbusch further pointed out that in blood within the vessels, the corpuscles usually run in the center of the stream, the periphery being made up of plasma alone (fig 4) Slowing or stasis of the circulation is followed by

DÉSIGNATIONS	HEMIA- TOBLASTES	HEMATES	GLOBULES Blancs
Nouveau-né de 24 heures	222 000	5 022 000	16 000
— de 3 jours	200 000	6 138 000	7 500
— de 6 jours	216 000	5 487 000	9 000
— de 8 jours	231 000	5 022 000	11 000
— de 13 jours	317 000	5 115 000	11 700
Enfant de 8 mois, non sevré	316 000	3 906 000	11 125
— de 8 mois, » »	276 000	4 960 000	21 740
— de 15 mois 1/2, sevré	318 000	3 006 000	10 000
— de 4 ans	260 000	5 177 000	12 000
Homme de 25 ans	231 000	5 487 000	5 200
— de 36 ans	231 000	3 979 000	6 830
— de 72 ans	318 000	5 091 000	7 700
Femme de 26 ans	231 000	4 557 000	5 300
— de 32 ans	220 000	4 274 000	6 600
— de 65 ans	216 300	5 763 000	5 650

FIG 5 TABLE GIVING THE RESULT OF THE FIRST ACCURATE PLATELET COUNTS BY HAYEM<sup>31</sup>

of blood generally accepted today as representing the normal does not differ significantly from that found by Hayem.

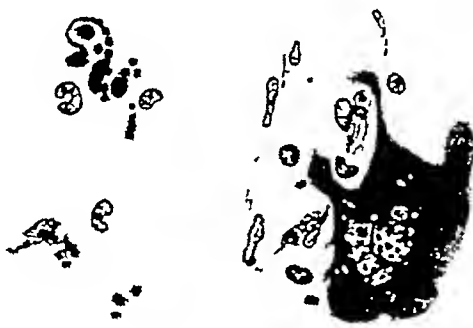
From a consideration of the facts brought out by Bizzozero, Hayem<sup>12</sup> concluded in 1882 that the platelet thrombus played an important part in the arrest of bleeding, and that a decrease or absence of platelets should result in disruption of the mechanism of hemostasis. A year later (1883) Krauss<sup>33</sup> reported in one form of hemorrhagic disease, purpura hemorrhagica, a diminution in platelets followed by an increase, after the hemorrhage ceased. In 1887 Denys<sup>34</sup> confirmed this finding and later Hayem<sup>35</sup> actually counted only 62,000 platelets per cmm of blood in a young patient with purpura. Hayem<sup>35</sup> <sup>36</sup> also drew attention to the large size of the platelets and the soft quality and poor retractility of clots formed from the blood of these patients, and attributed these defects to the decrease or absence of platelets.

Hand in hand with the efforts of observers of the time in affirming or denying the existence of the platelet as a distinct element, went attempts to trace the source of the elusive bodies. Many held the view that they originated either from nucleated red cells or from the red cells themselves. Engel<sup>37</sup> derived the platelets from the nuclei of normoblasts and Wlassov<sup>38</sup> and Bremer<sup>39</sup> believed they came from disintegrated erythrocytes, and did not accept them as constituting a third element of the blood. The bodies examined by Wlassov and Bremer must indeed have been by-products of disintegration of erythrocytes, resembling platelets only in form, the same bodies were later studied by Arnold<sup>40</sup> in intravascular clots and in blood allowed to stand outside the body for several hours. These ideas were corroborated and in part amplified by Muller,<sup>41</sup> Determan,<sup>42</sup> Maximow,<sup>43</sup> and Schwalbe.<sup>44</sup> Antagonists of the theory that the platelet was derived from the destruction of red blood cells were Petrone,<sup>45</sup> Sacerdotti<sup>46</sup> and Dominici,<sup>47</sup> who criticized the view of Arnold and adherents of his theories, and pointed out that their conclusions rested on artefacts from preparations of dried smears of blood. This recurring source of confusion was partly due to the fact that differentiating stains were, at the time, not generally available. It was not until the polychrome Romanowsky stains and azure dyes began to be widely used, that it was possible to separate the red-to-violet azurophilic granules of the platelet from all sorts of granular material in and out of cells. Dominici<sup>47</sup> introduced a new, and what proved to be a partially correct, conception of the origin of the platelet. He held that platelets were 'organites,' that is, formed elements liberated by cells and lacking a nucleus. As the 'mother cells' of the platelets, he described mononuclear cells with a protoplasm distributed in long pedicles which, when broken off, made up the platelets. It is possible that this conception influenced the work of Wright.<sup>48</sup> It differs from his theory in only one respect. Wright showed that this fragmentation took place from the cytoplasm of the megakaryocyte.

Two other workers of the Italian school came close to the solution of the problem of the origin of platelets. While studying the formation of the red blood cells, Foa and Salvioli<sup>49</sup> observed that the giant cell of the bone marrow, first described by Bizzozero<sup>50</sup> in 1869, and later (1890) called megakaryocyte by Howell,<sup>51</sup> fragmented into many colorless hyaline bodies. Foa and Salvioli thought these cell fragments were the precursors of the red blood cells. This view and Hayem's ideas

overlap at certain points, for Hayem thought that platelets (which we now know were the cell fragments observed by Foa and Salvioli) were the precursors of erythrocytes ('hematoblasts')

Bizzozero's conception of the platelet as an independent element of the blood was soon carried even further by those who claimed for it cellular characteristics common to most cells. Deetjen<sup>52</sup> using osmic acid fixation, believed he had shown that platelets had a nucleus and protoplasm. By special technics he also claimed to have demonstrated that platelets send out protoplasmic processes similar to the pseudopods sent out by leukocytes, a fact confirmed by Wright,<sup>48</sup> who saw some of the pseudopods retract. The observations of Deetjen were supported by Deckhuysen<sup>53</sup> and Koppsch.<sup>54</sup> These concepts influenced Deckhuysen perhaps to regard the mammalian platelet as homologous to the nucleated spindle cells of invertebrates and oviparous animals, which behave, when the blood is shed, very much



1906

FIG. 6. COPY OF A COLOR PLATE IN WRIGHT'S ARTICLE (1910)

Megakaryocytes bordering the sinusoids of the bone marrow and extending processes into the blood current, fragmenting giant platelets

like the mammalian platelet. This is why Deckhuysen called these cells in the lower species, *thrombocytes*.

Vassale<sup>55</sup> and Foa and Carbone<sup>56</sup> were among the first to find platelets in the spleen. Foa<sup>57</sup> differed from Vassale regarding their presence in the lymph nodes, Foa considered the hyaline bodies of the lymph nodes to be fragments of mononuclear cells and not true platelets.

Wright's discovery in 1906<sup>58</sup> that the megakaryocyte of the bone marrow gave rise to platelets by fragmentation of its cytoplasm was the next important development (fig. 6). All those present when Wright exhibited his preparations agreed that his demonstration was quite convincing.<sup>59</sup> His views, however, were taken up slowly by European investigators. Part of the reason for this might have been the relative lack of clarity of the illustrations accompanying his first contribution (1906).<sup>58</sup> His arguments were strengthened by a later report in which the documentation was clear and striking.<sup>48</sup> With but a few exceptions, one by one the leading histologists of the world have come to accept his views.

It was probably due to Wright's interest and stimulating influence that W. W.

Duke became interested in the problem of platelets and their relation to abnormal bleeding. Duke came under Wright's influence early in his career, as a house officer in the Massachusetts General Hospital. The role of the platelet in spontaneous hemostasis, first clearly visualized by Hayem, was convincingly shown by Duke<sup>60</sup> when he proposed the "bleeding time" of the skin as a test of a general tendency to bleed. Duke showed that there existed a close correlation between the number of platelets in the blood and the duration of the bleeding time. Apparently for the first time, abundant clinical as well as experimental evidence was gathered in support of this relationship. Duke was responsible, perhaps more than any other contemporary author, for strengthening the concept of the role and importance of platelets in disorders of hemostasis.

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## THE ROLE OF ALLERGY IN THE PATHOGENESIS OF PURPURA AND THROMBOCYTOPENIA

By FREDERICK W. MADISON, M.D.

ALLERGY by one term or another has been recognized as an etiologic factor in purpura since the earliest descriptions of that disease. Until very recently however, the intangible nature of both allergy and purpura, the multiplicity of potential etiologic factors, and the lack of exact knowledge of the pathologic changes in the latter state have made determination of the relative importance of allergy virtually impossible. Even now it is very difficult to evaluate the role of the allergic mechanism with accuracy. To attempt such an evaluation, it is essential to review briefly the chronologic development of the knowledge of the existence of allergic factors in the pathogenesis of purpura. It is likewise essential to define terms, for, as Piney<sup>1</sup> has recently stated, "Among the confused chapters in hematology, purpura is the most confused."

For a number of years it has been our custom to use the term purpura to indicate vascular changes only.<sup>2</sup> These vascular changes are characterized by reversible alterations of as yet unknown character in the walls of the smaller vascular radicles which make possible the escape of whole blood from the vascular bed. Such escape may occur more or less spontaneously (petechiae, nontraumatic ecchymoses) or it may be induced by increasing the intravascular pressure (e.g., tourniquet test,<sup>3</sup> Gothlin test,<sup>4</sup> etc.) or by decreasing the extravascular pressure (suction test,<sup>5</sup> capillary resistometer,<sup>6</sup> etc.). Artificial induction of petechiae is the most satisfactory criterion for the diagnosis of purpura at the present time, although lack of standardization of technics and variability of the vascular changes have made interpretation somewhat difficult. Other tests<sup>7-8</sup> are more complex and less suited to routine clinical use, but are useful for supplementary or corroborative purposes. After comparative trial of the various tests, we have come to rely almost entirely upon a simple tourniquet test done with a pneumatic arm band at 100 mm. of mercury (unless the systolic pressure is below that level, in which case it is correspondingly reduced) and maintained for 8 minutes, unless extravasation of blood is so marked as to cause excessive infiltration of cutaneous tissues, in which case it is stopped at a shorter time. A representative area 2.5 cm. in diameter is chosen, and the petechiae in it are either counted or recorded graphically. More than ten easily seen petechiae within the circle is regarded as a positive test, and the petechiae in excess of that number may be regarded as a quantitative expression of the test. A positive test is interpreted to indicate the presence of purpura, but a single negative test is not adequate to rule it out because of the variable nature of the vascular changes.

Purpura in this sense may and does most frequently exist alone.<sup>2</sup> It may, however, coexist with or be complicated by defects in the coagulation or clot retraction mechanism of the blood (hypoprothrombinemia, thromboplastin deficiency, thrombocytopenia, fibrinogenopenia) in which cases the combination of inadequate blood coagulation and "leaky" vascular walls produces an hemostatic error of such magnitude as to cause serious blood loss. From a clinical standpoint, it would seem to be much simpler to separate the vascular and hematologic factors and, at least for the purpose of etiologic studies, to consider thrombocytopenic purpura as purpura with thrombocytopenia or as two coexisting abnormalities. The im-



portance of this dual approach is apparent in tracing the chronologic development of the knowledge of the etiology of these states

One of the earliest discussions of the etiologic factors in vascular purpura is to be found in the "Opera Omnia" of Riverius. According to the English translation of Culpeper<sup>9</sup> (1678), he stated "But there is one Symptome proper and peculiar to a pestilential feaver which doth not happen in other Feavers, viz, Purple Specks or Spots on the whole body which the Italian Physicians name Peticulæ or Petechiæ, and these Feavers which have these symptoms are commonly named Purpuratæ or Petechiales and sometimes they are very large and possess whole members and then the parts appear tainted with redness which in a few hours oftentimes vanisheth away, and then returns again and are commonly called Ebullitions of the blood There do appear in other Diseases, spots very like unto those aforesaid, but springing from a far different cause, viz, from the over thinness of the blood, which being exagitated by the heat or the expulsive faculty does sprout forth of the Capillary Veins into the Skin These spots are wont for the most part to appear in such as have some Flux of the Blood, because the Blood in such is more thin and watery and also in Splenetick persons, and in such as have the Jaundice and old obstructions of the Bowels, and in all such who are apt to fall into a Cachexy " Interpreted in modern terms, Riverius suggested that infections, blood diseases, malignancy, diseases associated with splenomegaly, jaundice and cachexia were of etiologic importance in purpura The reference to the redness which in a few hours oftentimes vanisheth away, and then returns again" may well be construed to represent the earliest recognition of an etiologic mechanism which might now be regarded as of allergic nature

The next important addition to the list of causative or associated factors came a hundred years later when Werlhof<sup>10</sup> in 1735 described the classical case of an 'adult girl, robust, without manifest cause, attacked toward the period of her menses with a sudden severe hemorrhage from the nose and about the neck and on the arms, spots partly black, partly violaceous or purple " It is well to recall that no knowledge of thrombocytopenia or other coagulation defect existed in the eighteenth century but, regardless of the presence or absence of such defect, this would seem to be the first recognition of endocrine factors in the etiology of purpura In the same period Hornung<sup>11</sup> suggested that clinical purpuras be divided into simplex, febrile and scorbutic types, thus apparently recognizing in his third group what we now know to be deficiency states as important etiologic factors

The most tangible early suggestion of the relationship of allergy to purpura is found in the classification of Willan<sup>12</sup> (1808) which included five types purpura contagiosa, purpura simplex, purpura senilis, purpura hemorrhagica and purpura urticans It is of interest to note that this classification may have been the origin of the term purpura hemorrhagica which has continued in general usage to the present time A few years later Schonlein<sup>13</sup> (1837) reported the symptom complex which has borne his name and in which purpura occurred in association with multiple joint involvement The recent report of Montgomery<sup>14</sup> of the incidence of purpura in rheumatic fever has re-emphasized the importance of this symptom group In 1868 Henoch<sup>15</sup> described a similar case and recorded the addition of severe ab-

dominal pain and intestinal hemorrhage Six years<sup>16</sup> later he added several similar cases and the syndrome characterized by purpura and abdominal pain has since borne his name Many years later Glanzmann,<sup>17</sup> in reviewing these syndromes, suggested that the basic mechanism in all of the cases of both the Schonlein and the Henoch types was of allergic nature and suggested that they be termed "anaphylactoid purpura," a view which has been almost universally accepted The clinical reports of Osler,<sup>18</sup> and more recently of Eyermann<sup>19</sup> and others, have firmly established the importance of the allergic mechanism as an etiologic factor in a considerable portion of the cases of simple purpura without coagulation defect It is interesting to note that in 1914 Osler stated that 'perhaps the anaphylactic key will unlock the mysteries of the purpuras'

Thus by the middle of the nineteenth century the multiplicity of etiologic factors in purpura was well recognized It was known that it might occur as a result of or in association with a varied group of clinical states including infections, diseases of the blood, malignancies, cachexia, endocrine disturbances, deficiency states in addition to gastro-intestinal, skin and rheumatic syndromes that are now regarded as of allergic nature Clinical reports and experience since that time have confirmed amply the existence of all of these factors, and in recent years have emphasized the importance of the allergic group

Until the latter part of the nineteenth century, purpura was considered simply as a vascular disease because of the paucity of knowledge of blood coagulation defects There had been no knowledge of the existence of blood platelets until the studies of Donne<sup>20</sup> (1842) suggested the presence of a third cellular substance in the blood Hayem<sup>21</sup> confirmed these studies in 1878 and Bizzozero<sup>22</sup> completely established the identity of blood platelets in 1882 Brohm<sup>23</sup> (1881), Denys<sup>24</sup> (1887) and Hayem<sup>25</sup> (1895) soon discovered the fact that the blood platelets were sharply reduced in some cases of purpura though not in all

In the decade or two which followed the discovery of the relation of platelet reduction to purpura, profound changes occurred in the interpretation of the disease Attention was focused primarily on the platelet reduction, and clinical cases were divided into thrombocytopenic and nonthrombocytopenic types with subdivision into secondary and primary groups depending upon whether or not they were associated with recognizable clinical disease Partly because of its dramatic clinical manifestations, and partly because of the absence of tangible etiologic clues, primary thrombocytopenic purpura quickly occupied the center of attention and has retained that position to the present time Numerous synonyms have developed, including essential and idiopathic thrombocytopenic purpura and Morbus maculosis Werlhofii, with varying appropriateness It is important to recall also that, prior to the time when the concept of primary thrombocytopenic purpura came into existence, it was impossible to differentiate severe purpura from hemophilia, hypoprothrombinemia and fibrinogenopenia as we know them today Consequently, it is not surprising that thrombocytopenic purpura, with its severe and often lethal blood loss, should have been grouped with these diseases under the general title of hemorrhagic diseases and more or less separated from its closer allies, the nonthrombocytopenic or simple purpuras This confusion was rela-

tively short-lived however and was cleared by the development of practical methods for the enumeration of platelets, demonstration of the lack of syneresis in thrombocytopenic purpura (Hayem),<sup>25</sup> development of satisfactory methods for determination of coagulation time, development of the tests for bleeding time (Duke)<sup>26</sup> and prothrombin time (Quick),<sup>27</sup> and the clinical observations of Hayem,<sup>25</sup> Minot,<sup>28</sup> Duke,<sup>29</sup> and many others

The pathogenesis of the thrombocytopenia which was found in the last decade of the nineteenth century to occur so frequently in association with purpura has been the subject of many experimental and clinical studies since that time, although a few workers have maintained interest in the vascular phases of the disease, and a still smaller number have steadfastly persisted in efforts to correlate the vascular and hematologic phases. Hayem suggested that the reduction of platelets was due to decreased production or increased destruction of those elements, but since the origin and fate of the platelets were still unknown at that time he was unable to throw any light on either mechanism. Significantly, however, he did suggest the possibility of an allergic factor in the latter mechanism by demonstrating the reduction of platelets in 'anaphylactic' states after peptone injection, and with heterologous serum. He also demonstrated platelet reduction in severe infections. It was during this period, apparently, that the concept of the vascular changes being due to the platelet reduction gained favor, a concept which contributed much to the confusion of the subsequent years. Hayem's experimental work with heterologous serum doubtless provided the background for the tremendous interest in the reduction of platelets by the use of antiplatelet sera which was studied by many observers during the early part of the twentieth century and which demonstrated beyond question the susceptibility of the platelets to antiplatelet substances of biologic origin.

It was also during this period that the origin of the platelets in the megakaryocytes of the bone marrow was established by Wright<sup>30</sup> and corroborated by Bunting<sup>31</sup> and by Downey.<sup>32</sup> This observation provided a valuable clue to the mechanism by which platelet reduction might occur as a result of decreased production, and it was soon established by Duke,<sup>29</sup> Minot,<sup>28</sup> and others that platelet deficiency did occur in bone marrow diseases such as leukemia and aplastic anemia. Duke also confirmed the reduction of platelets in severe infections, notably diphtheria and tuberculosis, after peptone injection, after massive x-ray irradiation (Heineke),<sup>33</sup> demonstrated reduction by chemical toxins of the benzol type and, most importantly from the allergic standpoint, showed experimentally the reduction of platelets as a result of a hypersensitivity mechanism in rabbits sensitized to horse serum. Except for our present incomplete knowledge of deficiency of maturation factors and the role of the spleen, the etiologic background of thrombocytopenia was as complete in theory at that time as it is today. Myelopathy, severe infection and toxemia, chemical intoxication, x-ray irradiation, and allergic reaction had been established as important mechanisms capable of causing a reduction of platelets in the peripheral blood.

It was at that point that the tremendously important role of the spleen in platelet reduction was discovered more or less by chance. Kaznelson<sup>34</sup> demonstrated, and

many others have amply confirmed, prompt and dramatic increase of the circulating platelets following splenectomy in certain cases of thrombocytopenia. Interestingly, Elliott<sup>35</sup> has also demonstrated prompt reversal of the vascular changes following splenectomy in cases of purpura with thrombocytopenia. In the intervening thirty years splenectomy has been established as the standard therapeutic approach to cases of "primary" or "idiopathic" thrombocytopenic purpura, and has been shown to be particularly effective in those instances in which there is an ample number of megakaryocytes in the bone marrow. In spite of that position in therapy, however, removal of the spleen still remains a somewhat empiric procedure, for the mechanism by which it influences the level of the platelets in the circulating blood has never been clarified. There is still uncertainty and controversy as to whether the spleen destroys the platelets, inhibits their development in the bone marrow, or controls their release from the marrow, and what relation if any it bears to the allergic mechanism which seems to bear close resemblance to splenic action clinically.

Unfortunately, methods for the clinical study of the allergic mechanism in the production of thrombocytopenia, which has been so convincingly demonstrated experimentally, have been relatively unsatisfactory. However thrombocytopenia with purpura following ingestion of various drugs has been reported by Loewy,<sup>36</sup> Peshkin and Miller<sup>37</sup> and others, and thrombocytopenia has been produced at will in some of those patients by readministration of the offending drug and by skin testing, thus establishing the reaction as of allergic type. Further, demonstration of the allergic mechanism responsible for the granulopenia in agranulocytic angina,<sup>38</sup> and of the fact that sensitivity could be detected in those instances by granulopenic response following ingestion of the offending allergens, has suggested that platelets might behave in a similar manner and has corroborated the soundness of the "ingestion" method of demonstrating thrombocytopenic response to allergenic substances. Utilizing that method of testing in addition to the usual allergic diagnostic methods, it has been increasingly possible in recent years to establish allergic reactions as one of the causes of clinical thrombocytopenia with or without purpura. Squier and Madison<sup>39</sup> and others have shown that allergenic foods are capable of producing thrombocytopenia by demonstrating platelet reduction following ingestion of those foods, and have shown return of the platelet count to normal levels after the removal of the foods from the diet with clinical recovery.

Thus it is evident that in the evolution of knowledge of etiologic factors in thrombocytopenia and in purpura, allergy has been established as one of several factors capable of producing thrombocytopenia and likewise as one of several similar factors that are capable of producing the vascular changes characteristic of purpura. The curious similarity of these etiologic factors may well explain why purpura occurs so much more frequently in association with thrombocytopenia than with hypoprothrombinemia or thromboplastin deficiency. In the case of the allergic factor it is readily conceivable that an allergic individual might have both hematologic and vascular response simultaneously and to the same allergen, producing typical thrombocytopenic purpura. Clinical evidence to support this possibility is accumulating slowly, and is derived principally from satisfactory

clinical response of both thrombocytopenia and purpura to allergic control without splenectomy. With advances in the technic of allergic studies, and with wider use of the ingestion method of testing, it is likely that more cases will be found to fall into the allergic category and to respond favorably to that method of therapeutic approach.

### SUMMARY

It has been suggested that, for purposes of etiologic investigation, thrombocytopenic purpura be separated into its two component parts, thrombocytopenia and purpura, and that they be regarded as two coexisting abnormalities rather than as a single disease. Historical review of the development of knowledge of the pathogenesis of purpura emphasizes the importance and soundness of this dual approach. Both thrombocytopenia and purpura have been shown to have a complex etiologic pattern with multiple potential etiologic factors. The curious similarity of these two groups of factors may at least partially explain the frequent coexistence of the two abnormalities in the clinical picture of thrombocytopenic purpura.

It has been shown that allergy has long been recognized as an etiologic factor of major importance in both purpura and thrombocytopenia. It is logical, therefore, that it should frequently be an important etiologic factor when the two conditions exist together, and it is suggested that when diagnostic methods are more adequate a considerable number of cases of "idiopathic" thrombocytopenic purpura will fall into that category and will yield therapeutically to a proper allergic approach.

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# A STUDY OF THE BONE MARROW FROM THIRTY-SIX PATIENTS WITH IDIOPATHIC HEMORRHAGIC (THROMBOPENIC) PURPURA

By L. W. DIGGS, M.D., AND J. S. HEWLETT, M.D.

IN RECENT summary articles by Nickerson and Sunderland,<sup>1</sup> Rosenthal,<sup>2</sup> Tocantins,<sup>3</sup> Wiseman, Doan and Wilson,<sup>4</sup> Limarzi and Schleicher,<sup>5</sup> and Dameshek and Miller,<sup>6</sup> the extensive literature dealing with idiopathic hemorrhagic purpura is reviewed, and the known facts relating to the megakaryocytes and to the bone marrow are presented.

The majority of observations have been made on autopsy material, and there have been relatively few quantitative studies of material aspirated from the marrow.<sup>5-6</sup> General statements are often made concerning the value of the bone marrow examination in thrombopenic conditions, but specific facts are few. Because of the paucity of quantitative information, a lack of correlation of the marrow findings with prognosis, and differences of opinion concerning the number and morphology of megakaryocytes, a further study of the bone marrow seems justified.

In this paper the observations made at the Cleveland Clinic on the bone marrow smears of 36 patients with idiopathic hemorrhagic purpura, and the correlation of the findings with the clinical picture, are presented.

All of the patients had in common purpura, spontaneous bleeding from mucous surfaces, platelet counts below 100,000 per cu. mm., prolonged bleeding time, defective clot retraction, and normal or only slightly prolonged coagulation time. Smears from patients with demonstrable primary disease, leukemia, aplastic anemia, malignancy, nephritis, cirrhosis, or infections, or who gave a history of allergy or of taking drugs previous to hemorrhagic episodes, were excluded. Splenectomies, performed on 22 of the 36 patients, revealed normal or only slightly enlarged spleens. The tissue changes in the spleen were consistent with the diagnosis of essential thrombopenic purpura as defined by Nickerson and Sunderland. Unless specifically stated, all observations were made on marrow aspirated during the acute hemorrhagic phase of the disease.\*

The marrow was obtained by needle puncture of the body of the sternum in the midline at the level of the third rib. A minimal amount of marrow was aspirated, usually less than 0.2 cc. Smears were made directly from the point of the needle using the coverslip technic. The smears were stained with Wright's stain. Smears which contained relatively few nucleated red cells or early myeloid cells, and which were obviously diluted with peripheral blood, were not included in this study.

## THE BONE MARROW—GENERAL

The principal value of the bone marrow examination in thrombopenic purpura is to differentiate idiopathic hemorrhagic purpura from aplastic anemia, leukemia,

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\* Appreciation is expressed to Dr. Russell L. Haden, who performed the majority of sternal aspirations, preserved the smears for study, and who has allowed us to use the marrow and clinical material for this presentation.

and other conditions. In making this differentiation the megakaryocyte plays a part, but the other cells play a major role.

The bone marrow in idiopathic hemorrhagic purpura is cellular and there is little fat. The erythroid-myeloid ratio is normal in the majority of instances, but there is a tendency toward a relative increase in the nucleated red cells which sometimes approaches a 1:1 ratio. The distribution of nucleated cells in the bone marrow

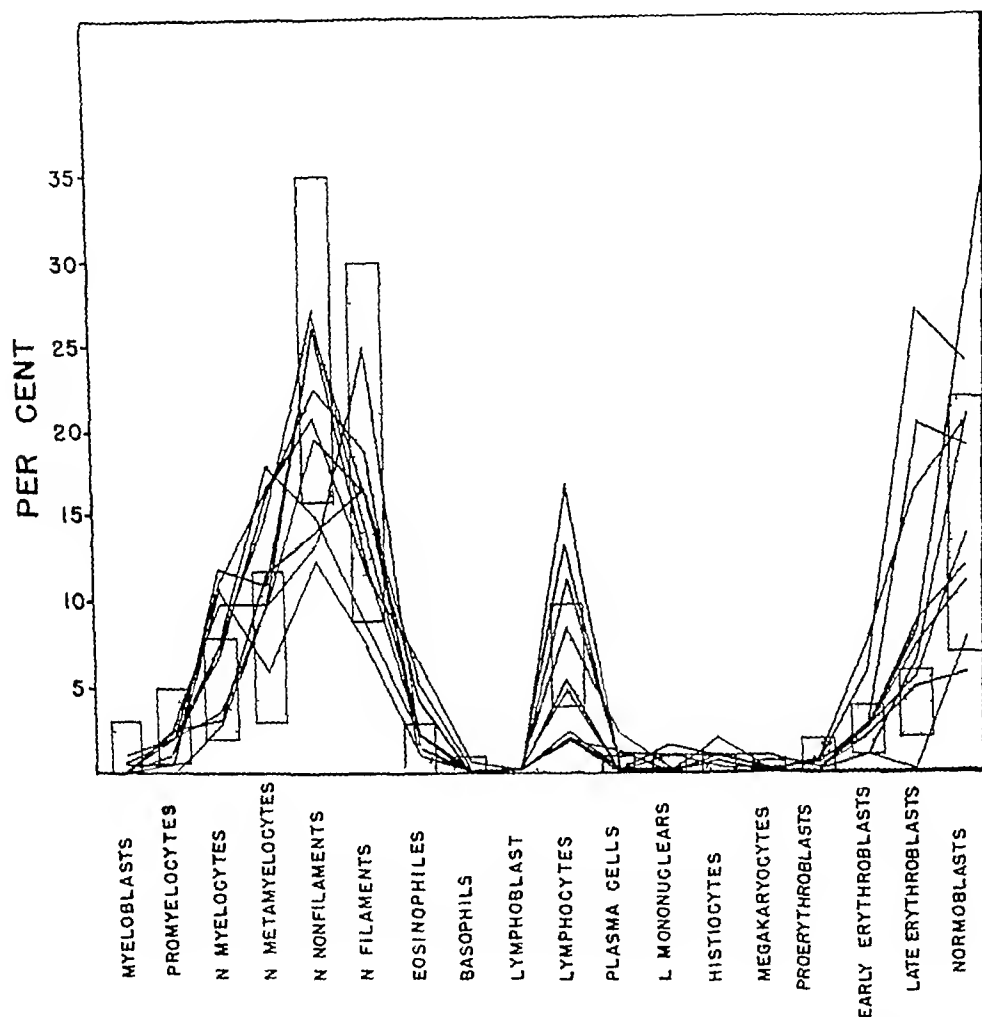


FIG 1 Frequency distribution of nucleated cells in marrow smears of 10 patients with idiopathic hemorrhagic purpura. The range of cells in nonhemorrhagic control cases is represented by the shaded areas.

smears of 10 of the patients of our series which is representative of the group as a whole is given in figure 1.

There is a slight left shift in the maturity of the myeloid cells with the majority of cells at the metamyelocyte to filament stage. The eosinophils and lymphocytes are usually normal, but may be increased. Each of the following cell types, undifferentiated primitive cells, histiocytes, mononuclears, plasma cells, and megakaryocytes, constitute less than 1 per cent of the nucleated cells. The nucleated red cells are mostly at the late erythroblast-normoblast level of maturation and appear normal in size, shape, and hemoglobin content. In patients with chronic purpura who are anemic as the result of blood loss, the nucleated erythrocytes



may be smaller than normal, their cytoplasm more basophilic, and their shape irregular. Platelets are difficult to find. Cells with vacuoles, toxic granules, and pyknotic nuclei or other signs of degeneration, are infrequent.

In aplastic anemia the marrow is relatively acellular. There is an absolute and relative decrease in myeloid and erythroid elements and a relative increase in lymphocytes, mononuclear cells, plasma cells and histiocytes. The lymphocytes and mononuclears are of a mature variety. There are few primitive cells. Megakaryocytes are difficult to find. The red cells reveal abnormalities in size and shape, and there are often atypical and degenerated leukocytes.

In smears from patients with leukemia there are numerous cells, usually of one variety. The normal marrow cells tend to be replaced. As a rule there are numerous primitive cells or "blasts." The megakaryocytes in leukemias at a stage in which there is purpura and thrombopenia are usually significantly decreased.

In our experience, the bone marrow examination has been of limited value in differentiating primary thrombopenic purpura from thrombopenia secondary to nephritis, Hodgkin's disease, disseminated lupus or malignancy. We have not had opportunity to observe a sufficient number of cases of purpura secondary to chemical poisoning or infections to draw conclusions, but degenerative changes in megakaryocytes and in other cells have been described in these conditions.<sup>7</sup>

#### NUMBER OF MEGAKARYOCYTES

The number of megakaryocytes in relation to other nucleated cells in smears of the bone marrow from normal individuals and from patients with idiopathic thrombocytopenic purpura as reported in the literature are summarized in table 1.

In our series, the megakaryocyte counts of bone marrow from 36 patients with idiopathic hemorrhagic purpura were made by noting the number encountered while counting 10,000 consecutive nucleated cells. All of the counts were made under high dry magnification or oil immersion. A tally counter was employed to facilitate counting. The number of megakaryocytes ranged from 3 to 59 per 10,000 nucleated cells, with an average of 17 (table 1).

Two sternal punctures performed on each of two patients within a period of three weeks, during which time there were active hemorrhagic phenomena, revealed megakaryocyte counts of 4 and 12 in one case, and 8 and 4 in the other.

In 50 bone marrow smears from patients with miscellaneous nonhemorrhagic conditions who had no evidence of blood dyscrasias and who had essentially normal marrows, the megakaryocytes ranged from 1 to 54, with an average of 16 per 10,000 nucleated cells. In this series the megakaryocytes were counted in consecutive fields, but the nucleated cells were counted in every tenth field until 1000 cells had been noted. The frequency distribution of the megakaryocytes in idiopathic hemorrhagic purpura and in nonhemorrhagic conditions is given in figure 2.

The number of megakaryocytes per low power field was estimated in 24 coverslip marrow preparations from patients with idiopathic hemorrhagic purpura. Twenty-five consecutive low power fields were examined in each case. It was found that the number per low power field varies from 0.0 to 8.2, with an average of 1.1.

TABLE 1—*The Range and Average Number of Megakaryocytes per 10,000 Nucleated Marrow Cells*

Observer	Method	Megakaryocytes per 10,000 nucleated cells						
		Control			Idiopathic Thrombopenic Purpura			
		Num- ber of cases	Range	Average	Num- ber of cases	Range	Average	Remarks
Nickerson and Sunderland	Marrow section	9 nor- mals	24-42	33	4	7-92	38	Postmortem
Limarzi and Schleicher	Indirect slide	10 nor- mals	—	0.59	5	1.4-13.8	7.1	Acute phase
					1	14.9		Chronic phase
Dameshek and Miller	Direct slide	10 nor- mals	0.99-2.7	1.82	5	3.7-7.4	5.2	Acute phase
					6	4.5-15.7	8.4	Chronic phase
Diggs and Hew- lett	Direct cov- erslip	50 misc condi- tions	1-54	16	36	3-59	17	Acute phase

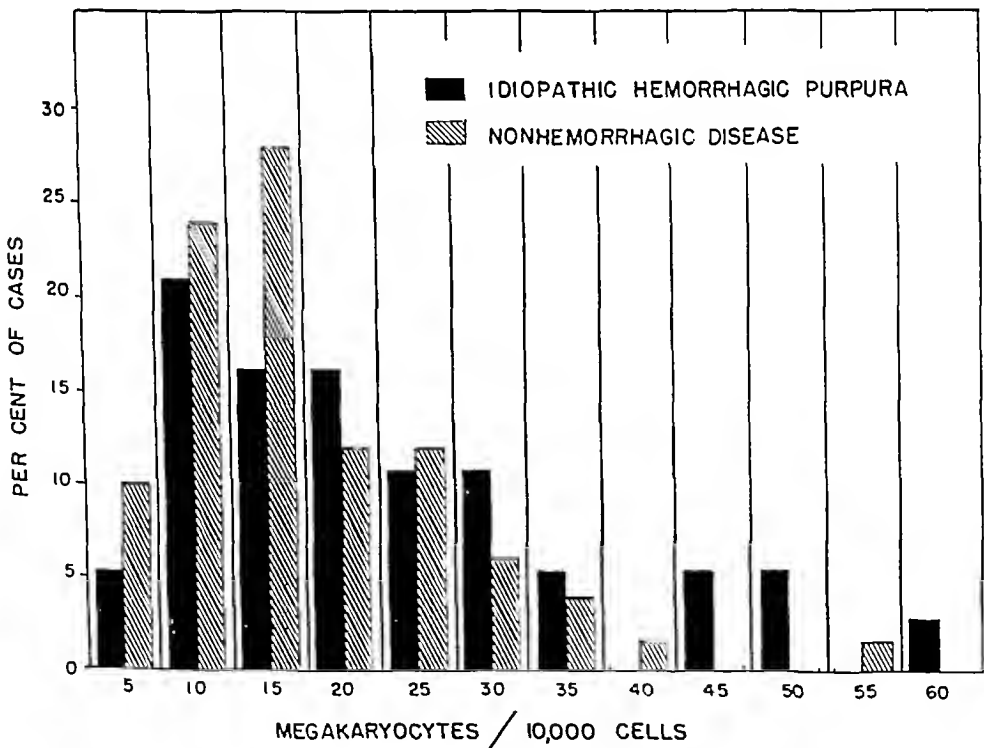


FIG 2 Frequency distribution of megakaryocytes per 10,000 nucleated cells in bone marrow smears from 36 patients with idiopathic hemorrhagic purpura and 50 patients with miscellaneous nonhemorrhagic conditions

Dameshek and Miller state that the average megakaryocyte count for miscellaneous conditions is just over 1, with a range from 0 to 5

Our observations agree with those found by previous observers that the number of megakaryocytes in idiopathic thrombopenic purpura is within the normal range, and that in some cases it is increased beyond the normal average. In several cases, not included in this series, in which the bone marrow smear was not considered satisfactory because of dilution with peripheral blood, the megakaryocytes were less than 1 per 10,000 nucleated cells, but in all preparations considered adequate for study megakaryocytes were readily demonstrable.

TABLE 2.—*Megakaryocyte Count in Relation to Postoperative Platelet Response and to Prognosis in Idiopathic Hemorrhagic Purpura with Splenectomy*

Megakaryocytes per 10,000 nucleated cells	Postoperative* platelet response (3 weeks)	Death	Recurrence	Cure
3		+		
4	++			+
5	+		+	
6	+			+
9	++++		+	
9	++			+
13	+++			+
14	++++		+	
16	++++		+	
16	++			+
17	+			+
19	++++			+
22	++++			+
24	++++			+
24	++	+		
24	+++		+	
29	†	+		
41	+++	+		
43	+++		+	
47	+++	+		
48	+++			+
59	++++			+

\* 500,000 or more platelets, +++++, 200,000-500,000 platelets, +++, 100,000-200,000 platelets, ++, less than 100,000 platelets, +  
† Operation performed elsewhere

THE RELATION OF THE MEGAKARYOCYTE COUNT TO PROGNOSIS

In order to test the truth of the common belief that patients with high megakaryocyte counts have a good prognosis and will respond favorably to splenectomy, whereas those with low counts have a poor prognosis, we arranged our patients according to the number of megakaryocytes per 10,000 nucleated cells and tabulated deaths, recurrences, and cures (tables 2 and 3). It is noted that there is no correlation between the number of megakaryocytes found in the marrow smears during the acute phase of the disease and the prognosis with or without splenectomy.

In patients with idiopathic thrombopenic purpura whose spleens were removed,

it was noted that there was a marked variation in the platelet response in different patients after operation (fig 3)

Of 22 patients subjected to splenectomy, 3 failed to show a platelet rise above 100,000, and 4 sustained a rise of from 100,000 to 200,000 platelets over a three-

TABLE 3 — *Megakaryocyte Count in Relation to Prognosis Idiopathic Hemorrhagic Purpura without Splenectomy*

Megakaryocytes per 10,000 nucleated cells	Death	Recurrence	Cure
6			+
8	+		
10			+
10	+		
13	+		
14			+
14		+	
20		+	
27			+
28			+
30	+		
35			+

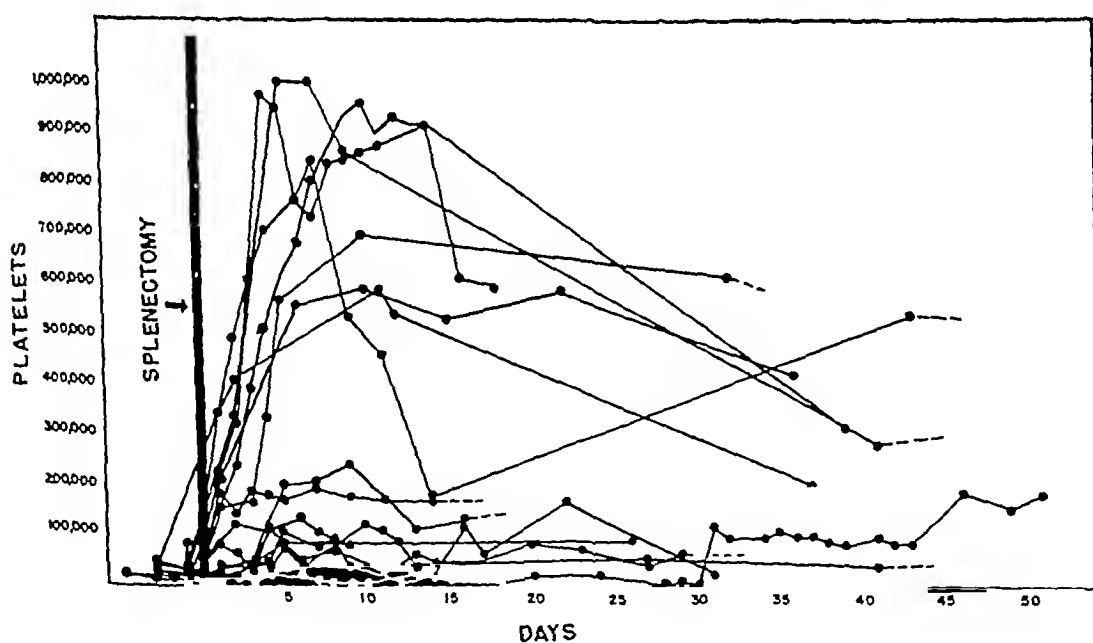


FIG 3 Platelet response following splenectomy in patients with idiopathic hemorrhagic purpura week postoperative period. Six patients responded with a platelet rise of 200,000 to 500,000, and 7 patients with a rise to 500,000 or more over the same postoperative period. There was no apparent correlation between the number of megakaryocytes and the platelet count following operation. One patient died soon after the spleen was removed. One patient was operated upon at another hospital.

#### THE MORPHOLOGY OF MEGAKARYOCYTES

The megakaryocytes were classified as megakaryoblasts, immature, intermediate,

or mature megakaryocytes, or as naked nuclei. The criteria used for the identification of each group are as follows:

1. *Megakaryoblast* (fig. 4 A, B). This cell has a diameter of 15 to 30 microns

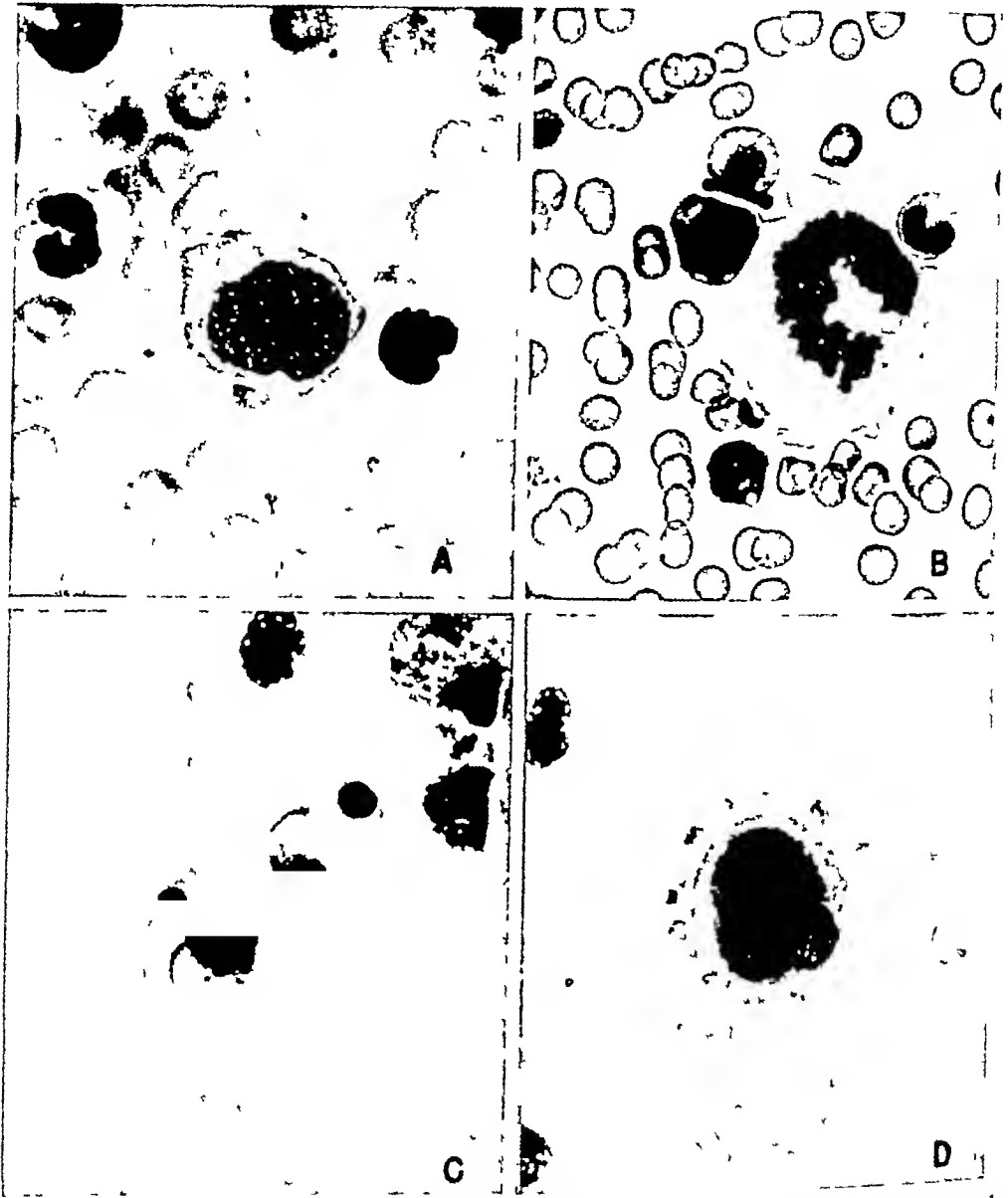


FIG. 4 A Undifferentiated stem cell (?) Megakaryoblast B Megakaryoblast in mitosis. Nongranular cytoplasm. Blunt cytoplasmic projections C Very immature megakaryocyte with two nuclei. Minimal amount of finely granular cytoplasm near the nucleus. Blunt nongranular pseudopods D Immature megakaryocyte. Finely granular cytoplasm. Multiple nongranular cytoplasmic tags

The shape is usually irregular and there are multiple blunt cytoplasmic projections. The nucleus is single, relatively large, round or slightly indented, and has a fine, lace-like chromatin structure which takes a predominantly acidophilic stain. Nucleoli may or may not be present. The cytoplasm is relatively small in amount, is nongranular, takes a basophilic stain and may have a spongy appearance.

2. *Immature megakaryocyte* (synonym promegakaryocyte, early megakaryocyte)

Fig 4 C, D, fig 5 E, F, G) This cell is larger than the megakaryoblast, being 20 to 50 microns in diameter. The shape is irregular, and there are varying numbers of cytoplasmic tags and rounded pseudopods. The nucleus is indented or divided

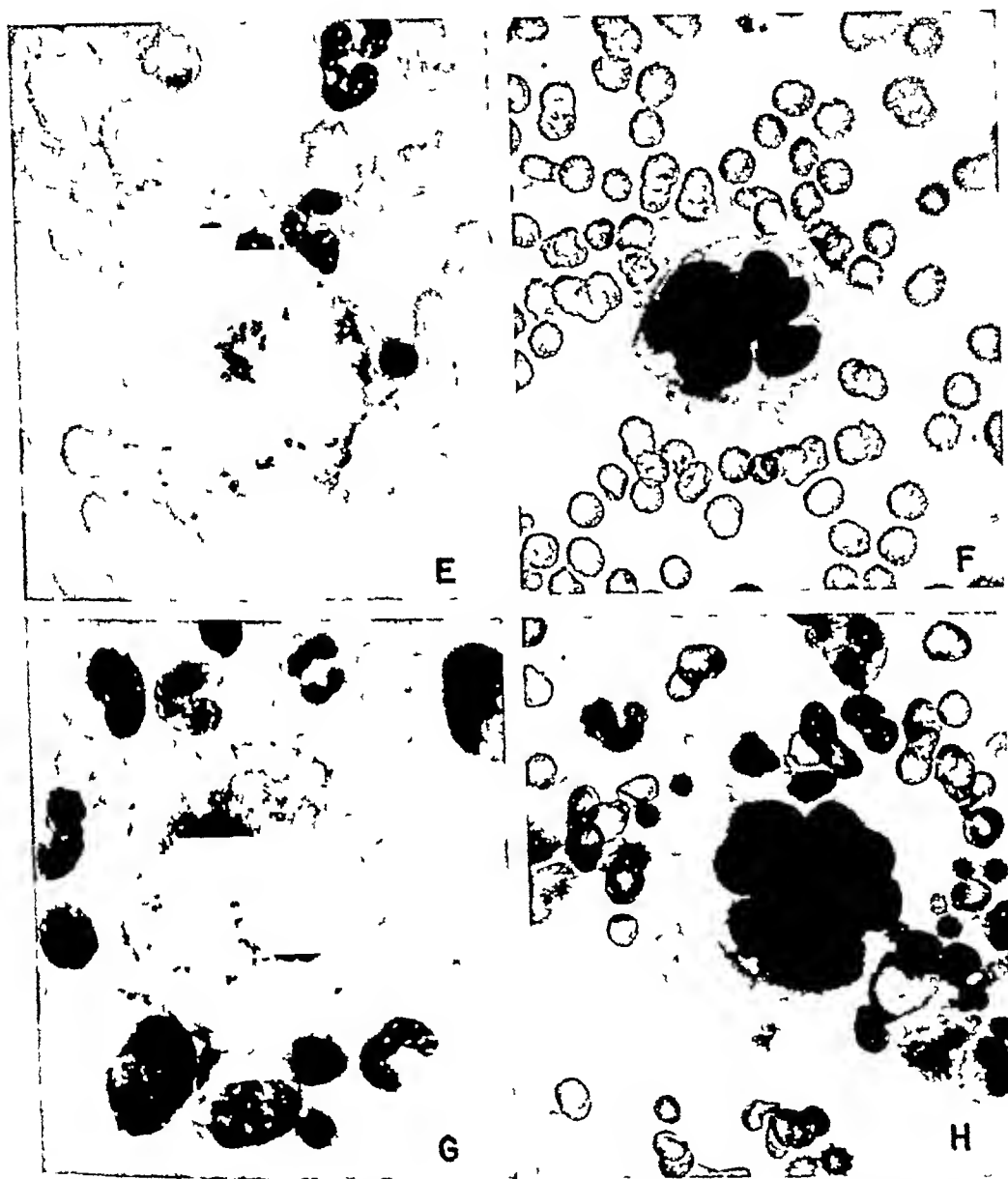


FIG 5 E Immature megakaryocyte with perinuclear granular cytoplasm. Spongy, nongranular peripheral cytoplasm and pseudopods. F Immature megakaryocyte with multilobulated nucleus. Granular area next to nucleus. Peripheral spongioplasm. G Immature megakaryocyte with granules next to nucleus, surrounded by a coarsely vacuolated zone and peripheral fine, spongy cytoplasm. H Intermediate megakaryocyte with ill-defined margins. Multilobulated, pyknotic nucleus. Granular cytoplasm. Absence of spongy layer at periphery.

into two or more lobes and has a moderately coarse chromatin structure without nucleoli. The cytoplasm stains blue and is likely to be darker near the nucleus than at the periphery. Fine bluish granules are demonstrable next to the nucleus, but granules are absent in the peripheral portions and in the pseudopods. The pseudopods often contain multiple small vacuoles.

3 *Intermediate megakaryocyte* (fig 5 H, 6 I, J) This cell is quite large, often measuring 50 to 80 microns in diameter. Its shape is usually round and the margins relatively smooth, but the edge may be frayed or there may be cytoplasmic tags

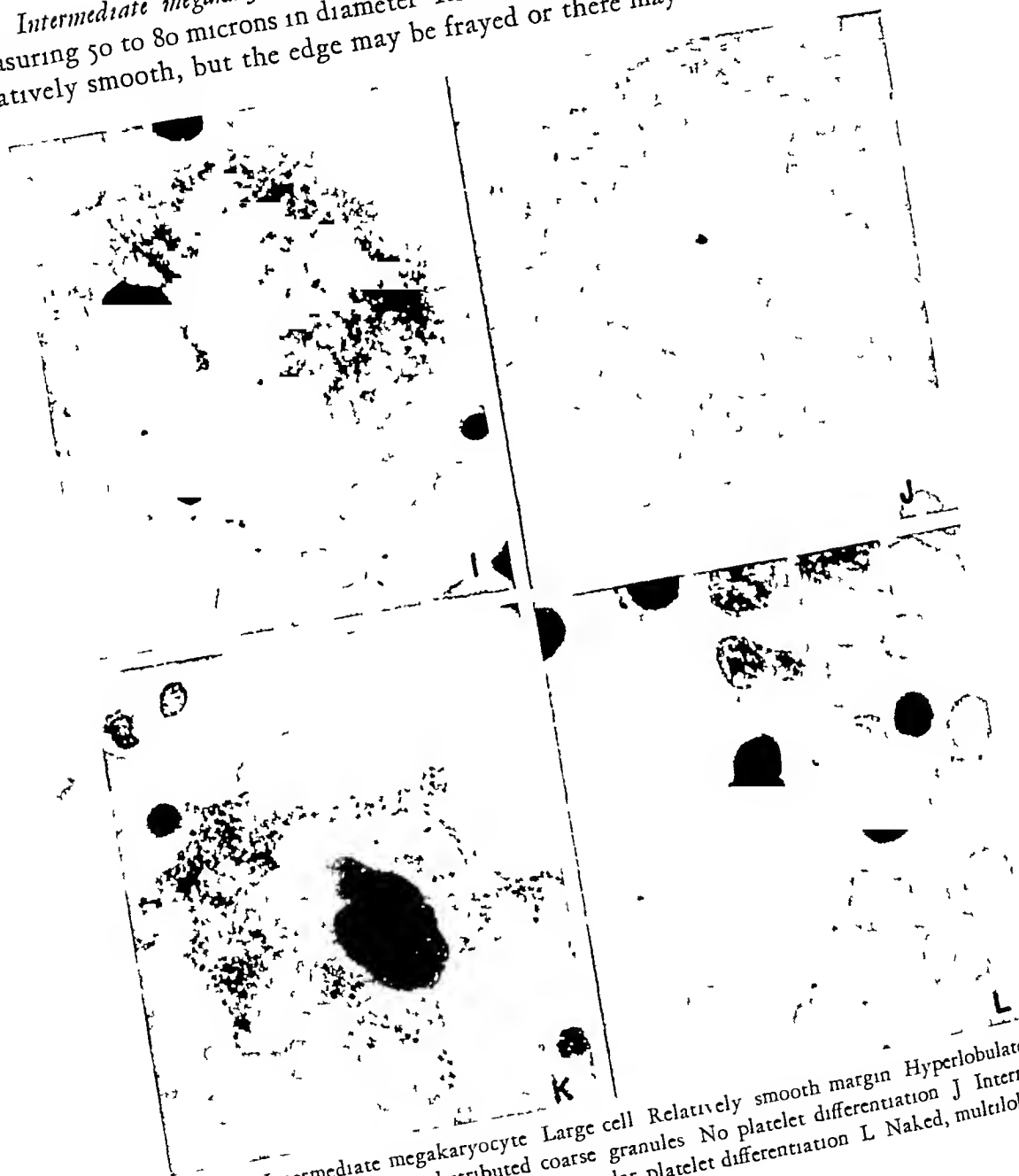


FIG 6 I Intermediate megakaryocyte Large cell Relatively smooth margin Hyperlobulated, pyknotic nucleus Fairly evenly distributed coarse granules No platelet differentiation J Intermediate megakaryocyte K Mature megakaryocyte Granular platelet differentiation L Naked, multilobulated, pyknotic nucleus

The nucleus is relatively small, is multilobular, and has a coarse chromatin structure. The cytoplasm varies in shade from blue to pink and is diffusely granular. The granules are relatively coarse. There may be vacuoles in the cytoplasm and a tendency toward a clumping of the granules, but there is no differentiation of well-defined granular platelets.

4 *Mature megakaryocyte* (fig 6 K) The mature megakaryocyte resembles the inter-

mediate cell in nuclear, cytoplasmic, and granular structures, but differs from it in that granular platelets are demonstrable. The platelets usually form small masses at the periphery, but the whole cytoplasm may be composed of granular platelet masses. The size of the cell varies widely from huge forms that practically fill the oil immersion field to fragments of nuclei with only a few platelets attached to them.

5 *Naked nucleus* (Fig. 6 L). In this form there is a pyknotic, lobulated nucleus and no cytoplasm.

There is often asynchronism in the maturation of nucleus and cytoplasm of megakaryocytes. Thus, one may have a multilobulated nucleus in a cell with basophilic nongranular cytoplasm or a single round immature nucleus in a relatively small cell which is actively producing granular platelets. Any cell which is producing granular platelets is called mature. The hyaline cytoplasmic projections and filaments and the spongy pseudopods are not considered as true platelets, but as artefacts, due to the tearing of the cell away from its fixed tissue connections or as evidence of amoeboid activity. Similar platelet-like structures are found in reticulum cells, malignant cells, plasma cells and in the early myeloid, erythroid, and lymphocytic cells.

It is to be noted that the principal criteria for the differentiation of megakaryocytes are the granules<sup>5-8</sup> and the presence or absence of granular platelets. The megakaryoblasts have no granules, the immature cells a few fine granules unevenly distributed, the intermediate cell has coarse granules fairly evenly distributed, but no well-defined platelets, and the mature cell has coarse granules and platelet formation.

One variant of the immature megakaryocyte (fig. 5 G) is a cell with a perinuclear granular cytoplasm, surrounded by a symmetrical ring of coarsely vacuolated cytoplasm and at the extreme periphery cytoplasmic attachments that have a nongranular hyaline or finely spongy character. This cell has been interpreted by others to be a degenerate form, but we consider it to be a form transitional between the immature and intermediate form. The well-defined spongioplasm probably represents an area of cytolysis which leads to a shedding of the peripheral cytoplasm and attachments to other cells, and to the production of a free granular cell which from that stage on produces true platelets.

#### THE DIFFERENTIAL MEGAKARYOCYTE COUNT

The differential megakaryocyte counts in our series were made by examining 25 or more megakaryocytes. For the control series, 50 smears of bone marrow from patients with nonhemorrhagic conditions who had no evidence of blood dyscrasias were examined. The distribution of the various stages of the megakaryocyte is given in figure 7.

In the control series the mature megakaryocytes which are actively producing platelets are the predominant cells, whereas in idiopathic thrombopenic purpura the intermediate cell without platelet production is predominant. In idiopathic thrombopenic purpura there is also a relative increase in immature forms and in naked nuclei.



The finding of reduced platelet formation and immaturity of cells is in agreement with the findings of numerous workers and confirms the original concept of Frank<sup>9</sup> and of Minot<sup>10</sup> that there is dysfunction of the megakaryocytes which prevents normal platelet formation

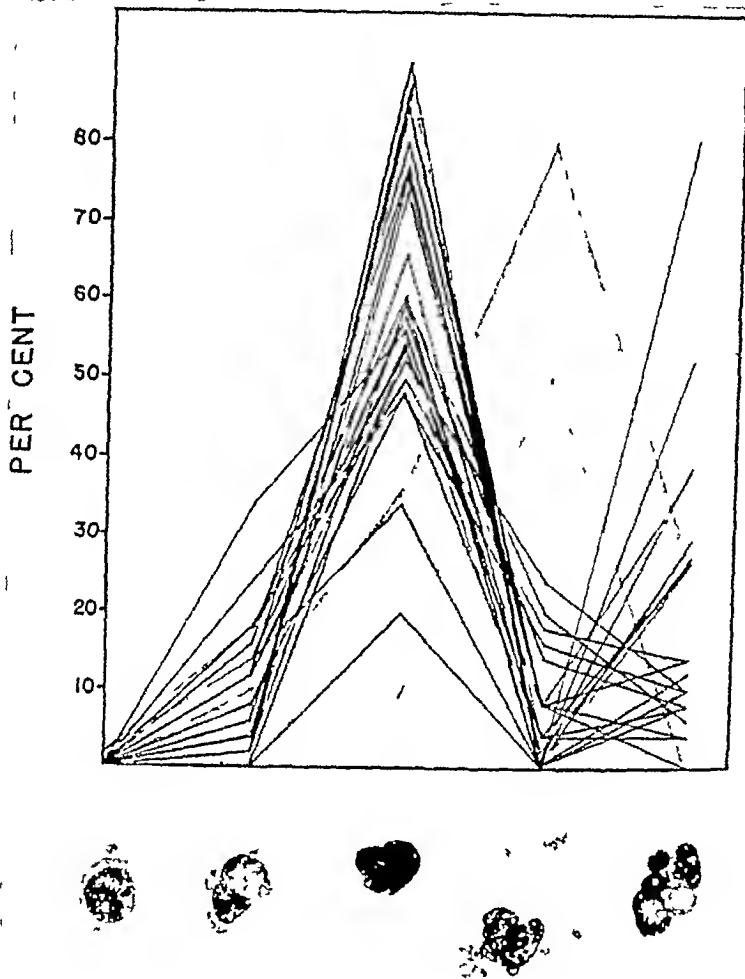


FIG 7 Frequency distribution of megakaryoblasts, immature intermediate and mature megakaryocytes, and naked nuclei in sternal bone marrow smears of 22 patients with idiopathic hemorrhagic purpura in the acute stage on whom splenectomy was performed. The rate of variation in nonhemorrhagic conditions is represented by the shaded area.

#### THE EFFECT OF SPLENECTOMY ON THE MEGAKARYOCYTES

Sternal marrow aspirations were performed on two of our patients before and after splenectomy (table 4). In both instances there was a decrease in the relative number of megakaryocytes after splenectomy and an increase in the mature platelet-producing cells. Similar decreases in the number of megakaryocytes have been noted by Wiseman, Doan, and Wilson, and by Limarzi and Schleicher. Limarzi and Schleicher, Dameshek and Miller, and others have also observed that following splenectomy there is a decrease in early forms and an increase in adult forms.

In one of our patients the marrow examination after splenectomy during a recurrence of the purpura revealed a megakaryocyte number and differential which was essentially the same as before splenectomy.

TABLE 4—*The Number and Distribution of Megakaryocytes in Smears of the Marrow Before and After Splenectomy*

Patient	Number per 10,000 nucleated cells	Megakaryoblasts	Immature	Intermediate	Mature	Naked Nuclei
M R 2 days before	48	0	16	76	6	2
6 days after	6	0	0	20	68	12
K D 2 hours before	17	2	18	54	0	26
43 days after	5	0	0	30	15	55

TABLE 5—*Prognosis in Relation to the Number of Eosinophils in the Bone Marrow (Idiopathic Hemorrhagic Purpura)*

Eosinophils per 1000 mature granulocytes	Deaths	Deaths during recurrence	Improvement with recurrences	Cure	Period of observation
With Splenectomy					
					175
21				+	2
30				+	1
31	+				
32				+	2
37				+	1
38		+			
39				+	6
39				+	1½
40				+	5
40				+	1
51			+		6
54	+				
60			+		4
61			+		2
71				+	½
77				+	4
80			+		¾
107	+				
110			+		2
140			+		1
158	+				
166				+	1½
Without Splenectomy					
0	+				
31				+	1
32				+	2
39				+	2½
50	+				
69	+				
77				+	
80				+	5
127			+		¼
156					2
191	+			+	6

## PROGNOSIS BASED UPON THE NUMBER OF EOSINOPHILS IN THE MARROW

Schwartz<sup>11</sup> in 1945 reported that in thrombopenic purpura "increased numbers of eosinophils in the marrow signify a favorable response for spontaneous recovery, while scant numbers foretell a chronic course and necessity for splenectomy." He made direct smears of material aspirated from the sternum. The number of eosinophils per 1000 granulocytes at the metamyelocyte stage or older were counted.

Schwartz interprets an increase in the eosinophils of the marrow as a manifestation of an allergic state or evidence of sensitivity to infections, drugs, or allergenic foods. He states that those with more than 50 eosinophils per 1000 granulocytes have an acute onset, a relatively benign course, and spontaneous and complete clinical and hematologic recovery and that removal of the spleen in such "allergic" thrombopenias is not necessary.

The relation of the eosinophils to prognosis was studied in our series, using the same method of counting eosinophils as recommended by Schwartz. No correlation was found between the eosinophil counts and the deaths, recurrences, or cures with or without splenectomy (table 5). We do not accept the thesis that eosinophil increase in the bone marrow is always indicative of sensitivity to exogenous food, drug, or other factors. There is evidence that the eosinophilia may be a reaction which is secondary to the hemorrhage into the skin or other tissues.<sup>12</sup>

## DISCUSSION

The variation in the number of megakaryocytes in different individuals with idiopathic thrombopenic purpura is in part due to true differences in individuals and in the distribution of bone marrow giant cells as has been shown in biopsy material by Lawrence and Knutti<sup>13</sup> and in autopsy material by others, but much of the variation is due to dilution of the marrow cells with peripheral blood and other technical factors. Megakaryocytes and particularly early forms are partially fixed cells which are not readily aspirated. These cells are fragile and easily destroyed by any manipulative procedure. They contain large amounts of thromboplastin and tend to get caught in fibrin webs which rapidly form around them. They are large cells which are pushed toward the margins and ends of slide preparations. We have found that the megakaryocytes in the best of coverslip preparations were unevenly distributed and that counts made on the same smears by the same or different individuals varies as much as 100 per cent.

Since there are unavoidable errors involved in megakaryocyte counting, a wide range of variation in different individuals, and within the range of observed values, no correlation between the megakaryocyte counts and prognosis, it is obvious that there is little use in undertaking the laborious task of making actual counts.

Prognosis and indications for splenectomy are determined, not from the megakaryocyte study alone, but from the study of the entire patient. If the diagnosis is aplastic anemia, leukemia or secondary thrombopenia, splenectomy is not indicated.

If, in 2 drops of material aspirated from the marrow (4 coverslip or two slide preparations), there are twenty or more megakaryocytes, if the majority of the bone marrow giant cells are immature or intermediate and not actively producing

platelets, and if the rest of the marrow, peripheral blood and clinical picture fits, the diagnosis of idiopathic thrombocytopenic purpura is made and splenectomy may be recommended

The finding of numerous megakaryocytes which are actively producing granular platelets is against the diagnosis of essential thrombopenic purpura or is indicative of a spontaneous remission in a known case. Splenectomy in such cases is contraindicated

The peculiar distribution of megakaryocyte types in some patients with essential thrombopenic purpura (fig 7) in which there are few mature forms, yet an increased number of naked nuclei, suggests that the intermediate cells disintegrate without going through the platelet producing stage. This is additional evidence that the low platelet count found in this disease is due to defective formation of platelets rather than to excessive destruction outside of the bone marrow

#### SUMMARY AND CONCLUSIONS

- 1 Observations made on the bone marrow smears of 36 patients with idiopathic hemorrhagic purpura and the correlation of the findings with the clinical picture are presented. The control series consisted of 50 patients with nonhemorrhagic conditions without blood dyscrasias
- 2 The bone marrow in idiopathic hemorrhagic purpura is hyperplastic. There is a slight myeloid and erythroid immaturity and in some cases a slight eosinophilia and lymphocytosis
- 3 The megakaryocyte counts ranged from 3 to 59 per 10,000 nucleated cells, with an average of 17. In the control series the megakaryocytes ranged from 1 to 54 per 10,000 nucleated cells, with an average of 16
- 4 There appears to be no correlation between the number of megakaryocytes found in the marrow smears during the acute phase of the disease and the prognosis with or without splenectomy. There is also no apparent correlation between the number of megakaryocytes and the platelet response following splenectomy
- 5 The megakaryocytes were classified as megakaryoblasts, immature, intermediate, or mature megakaryocytes, or as naked nuclei. The principal criteria for the differentiation of megakaryocytes are the granules and the presence or absence of granular platelets
- 6 The differential megakaryocyte counts were made by examining 25 or more megakaryocytes. In the control series the mature megakaryocytes which are actively producing platelets are the predominant cells, whereas in idiopathic hemorrhagic purpura the intermediate cell without platelet production is predominant
- 7 Marrow studies on 2 of our patients before and after splenectomy revealed a decrease in the relative number of megakaryocytes and an increase in the number of platelet-producing cells following operation
- 8 No correlation was found between the eosinophil counts and the deaths, recurrences, or cures with or without splenectomy
- 9 The principal value of the bone marrow examination in cases of suspected idiopathic hemorrhagic purpura is to exclude leukemia and aplastic anemia

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# THROMBOCYTOPENIC PURPURA COMPLICATING GOLD THERAPY FOR RHEUMATOID ARTHRITIS

REPORT OF THREE CASES WITH SPONTANEOUS RECOVERY AND ONE CASE WITH  
RECOVERY FOLLOWING SPLENECTOMY

By STACY R. METTIER, M D, ALICE McBRIDE, A B, AND  
JONAH LI, M D

IT IS A WELL established fact that certain drugs may cause thrombocytopenic purpura. With the introduction of the ever-increasing number of new drugs in the treatment of disease, this untoward reaction becomes more and more a medical problem. Mild to severe hemorrhagic tendency has been reported as occurring during the course of administration of sedormid, the sulfonamids, the arsphenamines, sodium salicylate, and other drugs. Recently an increasing number of occurrences of thrombocytopenia have been reported as resulting from the injection of gold salts for rheumatoid arthritis.

Hartfall, Garland, and Goldie<sup>1</sup> treated 900 patients with arthritis with injections of gold salts, and among this group there were 11 cases of purpura hemorrhagica. Three of these progressed to a fatal termination. There was no correlation between the severity of the platelet reduction and recovery or fatal termination.

Of the 245 patients treated with gold salts by Cecil, Kammerer, and De Drume,<sup>2</sup> 3 showed purpuric lesions in the skin. The lesions subsided when the drug was discontinued.

Price and Leichtenstritt<sup>3</sup> reported a series of 100 patients who had received gold salts, and 3 developed thrombocytopenia. Two of the patients recovered after withdrawal of the drug but the third died.

Short, Beckman and Bauer<sup>4</sup> treated a group of 47 patients with rheumatoid arthritis with injections of myochrysine in doses of 100 milligrams of the drug. One of their patients developed a severe hemorrhagic tendency with bleeding from the gums and nose. Platelets were not found present in the blood smear. In spite of repeated transfusions, the patient continued to show widespread bleeding, and death occurred five weeks after the onset of the purpura.

During the past seven years in the wards and out-patient department of the University of California Hospital, 160 patients, on whom a diagnosis of rheumatoid arthritis had been made, were given gold salt therapy. During the course of treatment 4 of the patients developed thrombocytopenia and purpuric manifestations. The results of the observations made on these 4 patients are reported.

## CASE 1

A W. A white American female, 44, was examined May 28, 1943. She stated that three years earlier she had first noticed a painful swelling of the middle joint of the third finger of the right hand. Since that time swelling accompanied by pain had appeared in other joints of the fingers and spread to involve the

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wrists, shoulders, elbows and ankles. In recent months she had lost considerable weight but was unable to estimate the amount.

*Physical examination* The patient appeared of small stature and structure. A survey of the skeletal system revealed a moderate degree of swelling of the interphalangeal joints of both hands. There was a soft fluctuant swelling over the dorsum of the right wrist. Both ankles were swollen and markedly tender to the pressure of the fingers.

*Laboratory data* Examination of the blood showed hemoglobin, 64 per cent (11 grams with standard at 17.2 grams per 100 cc), erythrocytes, 3,780,000 per cubic millimeter, leukocytes, 8800 per cubic millimeter, and platelets, 300,000 per cubic millimeter. Sedimentation of the erythrocytes was 37 millimeters in one hour. The plasma content of ascorbic acid was 1.7 milligrams per 100 cc and the uric acid was 3.3 milligrams per 100 cc.

*Diagnosis* Rheumatoid arthritis, hypochromic anemia.

*Course of the illness* The patient was returned to her physician for treatment with the advice that she receive injections of myochrysin in doses of 50 milligrams intramuscularly at intervals of one week. She was examined again December 15, 1943, at which time there had been a gain in weight of 6 pounds. She considered herself to be in good health and there was no apparent swelling of the joints previously involved. The hemoglobin was 116 per cent (Sahli) and the rate of sedimentation of the erythrocytes was 22 millimeters in one hour. The gold therapy was discontinued.

Three months later, March 11, 1944, the patient was admitted to the hospital because of recurrence of swelling of the joints and a moderate hypochromic anemia. Daily physical therapy consisting of radiant heat, massage, and passive exercise to the involved joints was given the patient. One month later, April 17, the patient received an injection of myochrysin, 25 milligrams in the buttocks. The following day a fine purpuric rash appeared and was widely spread over the body. Soon after there was moderate epistaxis on two occasions.

Examination of the blood showed erythrocytes, 3,050,000 per cubic millimeter, hemoglobin, 59 per cent (8.5 grams) Sahli. The white blood cells were 6650 per cubic millimeter. It was of interest that 3 per cent of the total leukocyte count was of the eosinophile variety. The blood platelets were 90,000 per cubic millimeter and there was a markedly increased fragility of the blood capillaries.

The patient was given a whole blood transfusion of 500 cubic centimeters. One week after its appearance the purpura had completely disappeared and the platelets had returned to normal numbers.

## CASE 2.

J. W. A white American man, 50, reported to the outpatient department of the University of California Hospital March 8, 1946, complaining of pain in the shoulders, elbows, wrists, hands, knees, and feet extending back over a period of ten years. Although he had carried on his usual occupation as a clerk, he had found it more and more difficult to get about because of progressive disability of the lower extremities and limited motion of the elbows and shoulders. Surgical removal of the thyroid gland had been accomplished at the age of 18 in an attempt to relieve hyperthyroidism complicated by exophthalmus.

*Physical examination* The arms were held rigidly to the patient's sides. With passive motion, practically no abduction of the right arm was possible, but elevation of the left arm to 20 degrees was accomplished with difficulty. There was mild, slightly painful swelling of the various interphalangeal joints, and there was thickening of the tissues on the dorsum of the wrists and about the knees and ankles. There was considerable atrophy of the muscles adjacent to the involved joints. An exophthalmus was still present as a residuum of the previously overactive and enlarged thyroid gland.

*Laboratory data* The blood count showed hemoglobin, 100 per cent (14.5 grams) Sahli, erythrocytes, 4,990,000 per cubic millimeter, and leukocytes 9960 per cubic millimeter. The platelets were reported to appear in an abundance on the stained films of blood. The blood uric acid was 3.5 milligrams per 100 cc. Sedimentation of the erythrocytes occurred at the rate of 33 millimeters in one hour. An estimation of the rate of basal metabolism was recorded at 18 per cent above normal. The plasma cholesterol was 300 milligrams per 100 cc.

*Course of the illness* On April 1, 1946, the patient was given his first injection of 50 milligrams of myochrysin (gold sodium thiomalate). The injections were repeated at intervals of one week accompanied by considerable improvement in the patient's well being. On December 17, 1946, the patient received his

twenty fourth injection which on this date made a total of 1200 milligrams of the colloidal suspension (myochrysine)

The patient failed to report for further treatment until seven weeks later, February 4, 1947, when gold therapy was resumed. He received the usual injection of 50 milligrams of myochrysine. The patient returned on February 10, stating that two days before minute areas of hemorrhage had suddenly appeared in the skin of the lower extremities and forearms followed some hours later by hemorrhagic blebs in the mucous membranes of the lips. That evening he passed a very loose stool that contained a considerable amount of bright red blood. The patient also observed oozing of blood from the gingival border of the gums.

*Laboratory data* Examination of the blood on February 10 showed hemoglobin, 90 per cent, erythrocytes, 4,000,000 per cubic millimeter, and the leukocytes 7500 per cubic millimeter. The blood platelets were estimated at 80,000 per cubic millimeter. A specimen of blood clotted at the end of eight minutes but failed to show retraction after several hours. The prothrombin content of the blood was 90 per cent of normal. Sternal puncture showed an essentially normal marrow picture with single cells and scattered islands of nucleated red cells and granulocytes. The megakaryocytes were increased in number but showed a greatly diminished platelet production.

Further injections of colloidal gold were discontinued and the patient was requested to report at frequent intervals for observation of the hemorrhagic tendency. On examination February 13, the hemorrhagic lesions had faded considerably. The platelets were 145,000 per cubic millimeter. The patient reported again February 17, when all evidence of hemorrhage had subsided and the platelets were 170,000 per cubic millimeter.

### CASE 3

S G A white American female, 33, was examined Nov. 5, 1946. Her illness started six years prior to entry, when she noted pain low in the back and the midthoracic region. Relief was obtained following the application of heat and the use of mild analgesics. Since then, there had been a recurrence of symptoms at intervals of one to two years. One year ago she became aware of pain in the cervical region and the gradual development of pain and swelling of the small joints of the hands. These symptoms have persisted since.

*Physical examination* The patient appeared slightly underweight and evidenced discomfort along the back. There was slight swelling of the first, second, and third metacarpophalangeal joints of both hands and of the proximal phalangeal joints of the middle fingers. Pain was elicited in those joints on pressure. There was stiffness of the cervical spine and forward flexion was limited to one-fourth the normal range. There was spasm of the paravertebral muscles in the lumbar region. Forward flexion of the lumbar spine was limited to three-fourths of the expected normal range. During this maneuver flattening of the spine became apparent in this region. No abnormalities were found on examination of the heart and lungs. Neither the spleen nor the liver could be felt.

*Laboratory data* The blood count showed hemoglobin, 69 per cent (11.9 grams), erythrocytes, 3,820,000 per cubic millimeter, and leukocytes, 6800 per cu. mm. The platelets were 350,000 per cubic millimeter. The plasma ascorbic acid was 1.9 mg. per 100 cc. The rate of sedimentation of the erythrocytes was 26 millimeters per hour. On examination of the roentgen films, narrowing of the cervical-dorsal interspace was apparent and the margins were sclerotic. The regional apophyseal joints, especially the eleventh dorsal, showed marginal sclerosis.

*Diagnosis* Rheumatoid arthritis of the spine and of the metacarpophalangeal joints.

*Course of the illness* Over a period of seven days, the patient was given a course of roentgen irradiation to the spine consisting of a total of 675 R. Four weeks later a similar course of treatment was pursued. Soon after, this was followed by complete relief from the back pain.

On Nov. 14, 1946, one week after the patient had been last exposed to the roentgen rays, she was given, intramuscularly, an injection of myochrysine 0.025 grams (gold sodium thiomalate). Three weeks later when the patient reported for the expected third injection she stated that for the past two days a fine red nonpruritic rash had been visible over the lower extremities. There was no bleeding from the gums.

*Laboratory data* The blood count on Nov. 27 showed hemoglobin, 81 per cent (11.68 grams) Sahli, erythrocytes, 3,930,000 per cu. mm., and leukocytes, 3250 per cu. mm. The blood platelets were greatly



reduced and showed 65,000 per cu mm on actual count. A specimen of blood failed to show clot retraction. When the Dalldorf apparatus was reduced to a pressure of minus 20 millimeters of mercury and applied to the skin of the arm above the antecubital fossa, it induced the appearance of large numbers of petechial hemorrhages. This indicated increased fragility of the capillaries. A sternal puncture showed an essentially normal marrow with the exception of a slightly increased number of megakaryocytes. These failed to show evidence of platelet production.

Further injections of gold compound were discouraged. One week later on examination the petechial rash had almost entirely faded and there was no evidence of new lesions. When the platelets were enumerated, there were 160,000 per cu mm and four weeks later, 200,000 per cu mm.

### COMMENT

The three case histories reported above are examples of thrombocytopenia arising secondary to intramuscular injections of a colloidal suspension of gold (myochrysine). This was accompanied by mild purpuric manifestations in two of the patients and was limited to petechial hemorrhages in the skin. In the third patient the hemorrhages were not only more extensively distributed over the body but were accompanied by bleeding from the nose and gastrointestinal tract. After three or four days without specific therapy, the blood platelets appeared spontaneously in the circulating blood in all of the patients in increasing numbers, coincident with the disappearance of the hemorrhagic tendency. At no time did the lives of the patients appear to be in jeopardy.

### CASE 4

R. B., a white American female, 44, was first examined Nov. 15, 1945. She stated that one year earlier she noted swelling of the small joints of both hands. Soon after, swelling, accompanied by pain, appeared in the wrists, elbows, knees, ankles, and small joints of the feet. During the past four months there had been a loss of 30 pounds in body weight.

*Physical examination.* On admission she appeared ill and underweight. Locomotion was accomplished with considerable pain and difficulty. There was marked pallor of the mucous membranes and of the palms of the hands. There was swelling of the middle interphalangeal joints of both hands, of the metacarpophalangeal joints, and at the wrists. There was slight ulnar deviation of the fingers. Extension of the forearms on the elbows was limited to 165 degrees. The shoulder joints were restricted to less than one half their normal range of motion. There was slight degree of swelling of the left knee and both ankles. A low pitched murmur was heard over the apex of the heart during systole and was thought to be of hemic origin. The spleen and liver could not be palpated.

*Laboratory data.* The blood count showed erythrocytes, 3,060,000 per cubic millimeter, hemoglobin, 38 per cent (6.5 grams) cenco, Sheard-Sanford electric photometer calibrated to 17.2 grams of hemoglobin per 100 cc blood, and leukocytes 7100 per cubic millimeter. The differential count was within normal range. The content of whole blood uric acid was 4.2 mg per 100 cc, and the plasma ascorbic acid was 0.45 mg per 100 cc. The hematocrit was 22.5 per 100 cc of blood. The sedimentation rate (corrected) was 10 mm per hour. The blood platelets were 220,000 per cu mm. The mean corpuscular volume was 70 cubic microns, the mean corpuscular hemoglobin concentration was 28 per cent, the mean corpuscular hemoglobin was 18 micrograms.

*Clinical diagnosis.* Rheumatoid arthritis, hypochromic anemia.

*Course of the illness.* On Nov. 20 and 23, the patient received transfusions of 500 cc each of whole blood which were followed by an increase in the hemoglobin to 60 per cent (10.3 grams) and in the red cell count to 3.74 M. Medication consisted of the oral administration of ascorbic acid 100 mg and ferrous sulphate 1.2 grams in divided doses daily. On Nov. 19, the patient received her first injection of colloidal gold (myochrysine, gold sodium thiomalate) 0.025 grams or 0.0125 grams actual gold. At the end of the fourth week the patient complained of a generalized itching rash which was erythematous in character. The injections of colloidal gold were discontinued. Two weeks later, Dec. 31, the patient returned stating

that the rash had disappeared. She stated that she was having much less pain, was more active and her general feeling of well being had improved. There was an increase in weight from 105 pounds at the onset of illness to 111 pounds on this date.

On Jan 21, 1946, the date she should have received her ninth injection of colloidal gold, there suddenly appeared some oozing of blood about the lower right premolar and there were innumerable petechial hemorrhages on the lower extremities. The blood count showed erythrocytes, 4,810,000 per cu mm, hemoglobin, 73 per cent (10.5 grams) Sahli, leukocytes, 11,100 cells per cubic millimeter, and platelets, 215,000 per cubic millimeter. Following application of the sphygmomanometer cuff to the arm, and elevating the pressure to just above diastolic, there was the appearance of petechial hemorrhages in the antecubital fossa. Chryso therapy was discontinued until on examination, March 18, 1946, the hemorrhagic tendency had completely subsided. On this date the hemoglobin had increased to 84 per cent but there were no significant changes in the erythrocytes or platelets.

Injections of colloidal gold were started and repeated at intervals of one week until July 8, 1946, when petechial hemorrhages reappeared in the skin. On this occasion the blood platelets were found reduced to 100,000 per cubic mm. The patient reported at intervals of one week until Sept 16, when she stated that she had had a severe epistaxis and, for ten days, a constant menstrual flow. Examination of the blood showed erythrocytes, 3,670,000 per cu mm, hemoglobin, 55 per cent, platelets 110,000 per cu mm, and leukocytes 7450 per cu mm. There were 5 per cent eosinophiles.

During the month of September the patient suffered frequent attacks of epistaxis, and on the occurrence of the menstrual flow the period extended over fourteen days instead of the usual four days. On two successive days she was given whole blood transfusions of 500 cc. Roentgen irradiation of the ovaries was resorted to as an attempt to induce artificial menopause. The menses failed to appear on the expected date, but bleeding elsewhere had become more profuse. On Oct 23, there was continuous oozing of blood from the nose and the gingival margins. Large ecchymoses appeared in the skin and large numbers of red blood cells were observed in the urine. An enumeration of the platelets revealed 10,000 per cu mm of blood. The bleeding time was greatly prolonged. A specimen of blood showed a tendency to coagulate after a lapse of ten minutes but the clot was soft, friable, and failed to retract. A sternal puncture showed marked erythropoietic activity as evidenced by large islands of nucleated normoblasts and erythroblasts. There was moderate myeloid hyperplasia. There was a slight increase in the number of megakaryocytes. These seemed rounded, somewhat opaque, and showed no evidence of platelet production. For the past week the patient had been given four transfusions of whole blood without materially altering the bleeding. It was apparent that the patient's condition was becoming critical and was approaching the state of purpura fulminans. On Oct 24, splenectomy was performed by Dr. Leon Goldman. The spleen was found enlarged to about twice its normal size. With the exception of a mild febrile reaction following a transfusion, the patient's postoperative course was without untoward event. The abnormal bleeding stopped. Four hours after the operation was terminated, the blood platelets were 210,000 per cu mm, twenty-four hours later they were 325,000 per cu mm, and three months later were 550,000 per cu mm.

#### COMMENT

The fourth case history reported here differed essentially from the preceding three in the duration and severity of the hemorrhagic tendency. Two months after the first episode of thrombocytopenia a second series of injections of gold salt was started only to be interrupted at the end of four months because of a recurrence of bleeding. For the next three months the patient continued to exhibit spontaneous bleeding of varying degree. Finally with the occurrence of frank hemorrhage from the mucous membranes it was apparent that there was an acceleration in the bleeding disturbance. Splenectomy was performed following which there was a complete disappearance of the abnormal tendency to bleed.

#### DISCUSSION

The cause for the thrombocytopenia which may occur during the course of gold therapy is not well understood. It is possible that it may be an allergic phenomenon.

wherein the megakaryocytes become so altered as to interfere with the production of platelets. In the three patients on whom sternal puncture was performed, it was observed that the megakaryocytes, although slightly increased in number, appeared to be deficient in platelet production. This reaction, however, is not unique for the thrombocytopenia that occurs in gold therapy, since various investigators, including Frank<sup>5</sup>, Limarzi<sup>6</sup>, and Dameshek and Miller<sup>7</sup> have described the apparent inactivity of the megakaryocytes in cases of so-called idiopathic thrombocytopenia.

In recent times the term "hypersplenism" has appeared in the medical literature. It is presumed that an abnormal spleen acts upon the bone marrow, or more specifically the megakaryocytes, to produce an inhibitory mechanism. Emphasis for this postulation is found in the greatly increased production of platelets soon after splenectomy.

The management of a patient with thrombocytopenia induced by gold salt therapy offers a serious problem to the clinician. In most instances the abnormal bleeding is of short duration. On the other hand, the bleeding may become enhanced and prolonged, may fail to respond to transfusions and ultimately lead to the patient's death. The question arises as to whether splenectomy should or should not be performed. It has been the consensus of opinion among surgeons<sup>8</sup> that "splenectomy is not indicated but rather contraindicated in secondary thrombocytopenic purpura due to severe infections or intoxications." In none of the other reported cases of thrombocytopenia secondary to gold had splenectomy been attempted. In the present case the mild hemorrhagic disturbance of four months' duration became suddenly accelerated by the appearance of marked bleeding from the mucous membranes. It was felt that since the life of the patient was in danger, splenectomy was advisable. Soon after the operation there was a dramatic response of the platelets which led to the recovery of the patient.

Of interest in connection with the case reported here is the one reported by Farfel,<sup>9</sup> wherein a patient with thrombocytopenia due to sulfathiazole therapy recovered following splenectomy.

#### SUMMARY

Of 160 patients treated with gold salt therapy, four developed thrombocytopenia with mild purpuric manifestations. In three patients there was a spontaneous remission and the hemorrhagic tendency disappeared in about one week's time.

In the fourth patient there was a second occurrence of thrombocytopenia which persisted over a period of four months, and finally failed to respond to transfusions. Splenectomy was followed by a dramatic rise in the blood platelets and recovery of the patient.

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## INCONSTANCY AND VARIABILITY OF THE VASCULAR FRAGILITY TEST EVEN IN PURPURIC CONDITIONS

By J. ROSKAM, M.D., CH. RENARD, M.D., AND L. SWALUE, M.D.

THE PATHOGENESIS of purpura is mysterious and complex. This is true for the origin of uncontrollable hemorrhages in Werlhof's disease and similar conditions. Clinical and experimental observations have indeed proved that the very long bleeding times occurring in hemogenic and hemophilo-hemogenic syndromes are due to the association of a hemic and a vascular factor (Roskam, <sup>11-12</sup>). Although the existence of the latter factor is established beyond doubt, its nature is not yet completely elucidated.

The breaking out of intradermal purpura seems also to result frequently from multiple factors (Grenet<sup>4</sup>, Bedson<sup>1</sup>), seldom analyzed as yet.

Several authors have, however, emphasized the importance in the production of petechiae of the fragility of vessels of purpuric patients, as namely evidenced by the ease with which venous stasis produced a petechial eruption in these patients.

This induced purpura was described in 1911 by Frugoni and Guigni,<sup>2</sup> later by Weill (of Lyon) and Chalié<sup>16</sup> in certain hemorrhagic conditions. The same year, Leede<sup>5</sup> erroneously considered it as pathognomonic of scarlet fever. Later this phenomenon was systematically studied and was found by many authors in various conditions: intoxications, avitaminoses, diseases of the endocrine organs, of the spleen, the reticulo-endothelial system, of the sympathetic nervous system (Stephan<sup>16</sup>), in hypertension, solitary or combined with arteriosclerosis, in nephritis, in endocarditis lenta (Weissman<sup>17</sup>), in diabetes, in rheumatic patients given large amounts of sodium salicylate, in patients with gastro-duodenal ulcers treated with a milk diet and alkaline powders, in patients with chronic glaucoma (Roskam<sup>9</sup>), in erythremia, icterus, certain forms of tuberculosis (Schour<sup>14</sup>), etc.

The intensity of the purpura induced by venous stasis, "signe du lacet," capillary fragility test, capillary resistance test, or tourniquet test, etc., is approximated by the number of petechiae which appear during the test.

One of us has, however, repeatedly pointed out the "qualitative" and not "quantitative" aspect of the eruption, i.e., the importance of the size of the different purpuric elements. Petechiae with a diameter above one millimeter possess a significance similar to that of increased bleeding time (Roskam<sup>10</sup>).

These proportionally large petechiae are observed in subjects with a hemorrhagic condition. The more severe the condition, the larger is generally the diameter not of all petechiae, but of a certain number of them.

After this brief review, we will take the opportunity of describing two recent clinical observations to underline the diverse results in different circumstances of the "vascular" fragility test, as we prefer to call the "capillary" fragility test, for no one has proved that only capillaries are involved in that test.

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## CASE HISTORIES

*Case 1* V Michel, male, 16, high school student

*Family history* irrelevant

*Personal history* measles, scarlet fever, diphtheria, appendectomy in childhood

*Present disease* At the beginning of December 1946, appearance of an eruption not disappearing by vitropression, made up of small elements grouped in clusters on the antero-medial aspect of the forearms and thighs. Later extension of this eruption mainly to the shoulders, to the anterior side of the legs, and to the ankles. Purple at their appearance, the small eruptive elements turn later brown-pink and yellow before disappearing. Several eruptive waves follow, always occurring in the skin areas involved in the first attack.

*Physical examination* On Jan 10, 1947, nothing unusual, except for pigmented sequelae of previous eruptions and for a few small submaxillary and axillary nodes.



FIG. 1

*Sedimentation rate* 3 mm in the first hour (Westergren)

*Hemoglobin* 55 per cent

*RBC* 4,200,000 *Morphology* normal

*WBC* 7,200

*Differential* Neutrophilic polymorphonuclears, 48 per cent, eosinophilic polymorphonuclears, 3 per cent, lymphocytes, 43.5 per cent, monocytes, 5.5 per cent

*Platelets* 220,000 *Morphology* normal

*Bleeding time* Right ear, 1'30", 1', 1', 3'30", 2' Left ear, 1'30", 1', 1'30", 2'30", 1'30"

*Clotting time* 22', 22', 22', 22' (normal 16' to 24')

*Prothrombin time* (Quick) 100 per cent

*Border-Wasserman, Meinicke, Paul and Bunnel reactions* negative

*Vascular fragility test*\* quantitative, ++++ qualitative, +++, only in the cutaneous areas corresponding to previous eruptions, as the petechiae induced by venous stasis are grouped in clusters and form eruptive blotches separated by areas of normal or almost normal skin (figs 1, 2, and 3)

One week later, despite the daily intake of 40 mg of citrin, similar vascular fragility test quantitative, ++++, qualitative, +++ in the cutaneous areas with sequelae of previous eruptions, almost negative between these areas

*Case 2* Jacques, male, 17, high school student

*Family history* irrelevant

*Personal history* measles, chicken pox, mumps, whooping cough in childhood, appendectomy in 1941, frequent bronchitis, gastro-intestinal upset with fever in October 1946

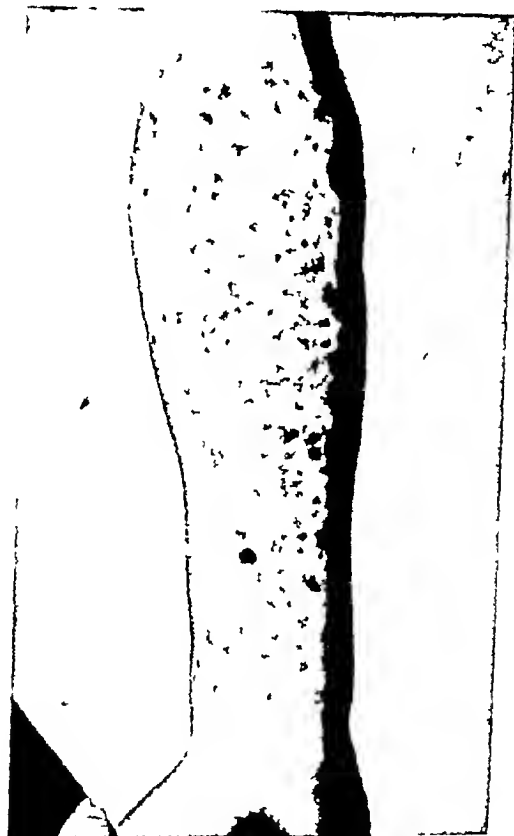


FIG 2

*Present disease* On Saturday, January 25, 1947, the patient ate lobster with cocktail sauce, duck, and *pâte de foie gras*. Normal activity from January 26 to 28, but exposure to very cold weather during these three days. On Tuesday 28, purpuric stripes (length 1½-7 cm, width ½-2½ cm) suddenly appeared on the face and neck. On the 29th, fever (about 38°C) which lasted for two days, headache and nausea, whereas the eruption faded out progressively and disappeared on Feb 10.

On Feb 2, at 8 P M, intake of two boiled eggs and again exposure to severe cold. At 9 P M, as his

\* The vascular fragility test which we used is the test described by one of us in 1929 under the name of "signe due brassard" application above the elbow of the cuff (brassard) of Bouliette's oscillogometer inflated half-way between the maximal and minimal arterial pressure of the patient. The pressure is held for 15 minutes. After decompression, there is examination of the induced purpura on the whole surface of the forearm and hand, and not, as later proposed by Wright and Lilienfeld,<sup>18</sup> in a small area of the supero-medial aspect of the forearm.



FIG 3



FIG 4



father was weighing and measuring the patient, he saw the appearance of a new eruption which reached its maximum in a quarter of an hour. More numerous, the purpuric spots were localized on the face, neck, upper part of the chest, forearms, and wrists. Soon afterwards, fever (38.2 C), headache and nausea.

*Physical examination* On Feb. 3, 1947, in addition to the cutaneous eruption (figs. 4 and 5), there were slight fever (37.5-38.5 C) which disappeared progressively in ten days, a few petechiae on the soft palate, a few small nodes in the neck, groins and axillae, and a palpable spleen reaching the costal margin on percussion.

*Sedimentation rate* 4 mm in the first hour (Westergren)

*Hemoglobin* 100 per cent

*R B C* 4,750,000 *Morphology* normal

*W B C* 6,000

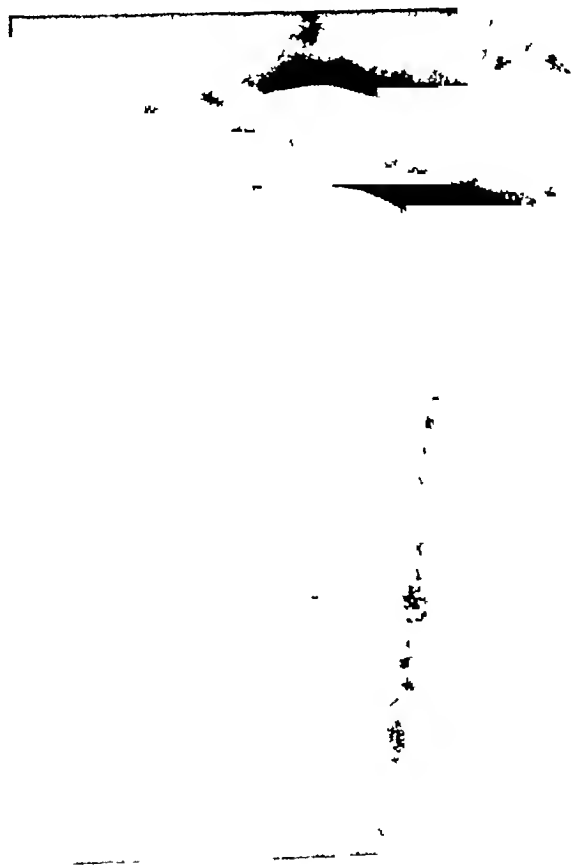


FIG. 5

*Differential* Neutrophilic polymorphonuclear, 60, eosinophilic polymorphonuclear, 2, lymphocytes, 31, monocytes, 7

*Platelets* 225,100 *Morphology* normal

*Bleeding time* Right ear 1', 1', 1 30", 2', 3' Left ear 3', 2', 1', 1', 2'

*Clotting time* 21', 23', 21', 23' (normal 16' to 24')

*Prothrombin time* (Quick) 100 per cent

*Bordet-Wasserman, Mechnik, Widal, Wright's reactions* negative

*Paul and Bunnell's test* + at 1/8

*Hemoculture* negative

*Takata-Dohmoto's flocculation figure* 59 (low, but still normal)

*Weltman's coagulation band* 0.4 (normal)

*Vascular fragility test* quantitative and qualitative practically negative on this day, as well as later, when the eruption was fading or had disappeared

As the history and tests suggested an anaphylactic purpura, preparations of egg white, mayonnaise, foie gras, and lobster were scratched into the skin no reaction. Similarly, intradermal reactions with milk, tuberculin, Dmelcos, antistaphylococcic vaccine remained negative, as well as attempts to find a possible focal infection. The intake of foie gras on Feb. 20 did not bring any of the reactions characteristic of Vidal's digestive hemoclasia, nor any rash.

However the patient—who had been kept at normal temperature—ate on Feb. 6, at noon, lobster cocktail sauce and, in the evening, some foie gras. On the next morning, the purpuric stripes appeared at the base of the neck and on the anterior aspect of the forearms, the latter spots were surrounded by an area of congestion without pruritus. The eruption induced on February 6 and 7 was much milder than the previous ones. This was the last of the purpura of this patient.

On Feb. 14, Paul and Bunnell's reaction:  $1/16+++$ ,  $1/32++$ ,  $1/64+$

### COMMENTS

Both cases reported had a purpuric eruption.

In the first one, the eruption was symmetrical and made up of innumerable petechiae grouped in cluster, in maplike areas 0.5 to 2 cm. in diameter, separated by areas of almost normal skin. This relapsing purpura simplex had a protracted course or at least a subacute one. The etiology remained mysterious, as well as the cause of the different attacks. However, outside any purpuric attack, the vascular fragility test was twice strongly positive, quantitatively and qualitatively, but only in those cutaneous areas where the previous eruptions had spontaneously occurred.

In the second patient, the eruption, also symmetrical, was formed by rather homogenous hemorrhagic streaks not resulting from the coalescence of smaller petechial elements. The only real petechiae were observed on the soft palate. The etiology of the syndrome probably was alimentary. However, it is noteworthy that the cuti-reactions with the suspicious foods remained negative, as well as Vidal's tests of digestive hemoclasia, and that the purpura simplex of this patient occurred at the same time as an attack of infectious mononucleosis. The vascular fragility test was completely negative at the place of the purpuric spots and outside them.

Thus, in one of the two reported cases of purpura simplex, a strongly positive quantitative and qualitative vascular fragility test was present.

In the other, the vascular fragility test was completely negative.

In order to explain the different behavior of the vessels of the two patients during venous stasis, one might consider the different nature of the two cases of purpura, as also evidenced by differences in the clinical course and in the purpuric eruption.

This simple interpretation is probably accurate.

Nevertheless a case of constitutional athrombopenic hemorrhagic purpura published by one of us in 1929 (Roskam<sup>8</sup>) might be taken as an argument against it.

For this patient we noted on April 5, 1927, at the time of his admission to the hospital:

Tourniquet test after 15 minutes of compression at 70 mm. of mercury with the blood pressure machine: there appeared below the right elbow several large petechiae measuring 1-3 mm. About 50 were seen on the anterior aspect, 40 on the posterior aspect of the forearm. Under the same conditions, the test being repeated twice, no purpuric elements were seen over the left forearm. On April 29 we noted

"Tourniquet test after 15 minutes of compression of the arm at 80 mms of mercury, identical numbers of petechiae appeared over the right forearm as had been noted on April 5. The left forearm at this time showed a very marked purpuric eruption with enumerable punctate petechiae. 195 petechiae were present on the anterior aspect of the left forearm together with 6 ecchymoses of about 5 mms in diameter. Posteriorly 175 petechiae of about 1 mm in diameter were seen. Tourniquet test after 15 minutes of compression at a pressure of 100 cu mms of mercury, an intense purpuric eruption about the same both in the right and left arms occurred. Numerous petechiae were seen. These extended over the dorsum of the hands and over the fingers."

We have thus observed a case of constitutional athrombopenic hemorrhagic purpura with, during an acute period, a vascular fragility test quantitative +, qualitative ++ on the right side, completely negative on the left side, a few days later, quantitative +, qualitative ++ on the right side, quantitative and qualitative +++++ on the left. Two years later, without hemorrhagic episode, the vascular fragility test was strongly and equally positive on both sides.

This observation indicates the great variability of the vascular fragility test at different times, and also at different sites, in symmetrical areas of the skin in a case of chronic hemorrhagic purpura.

Together with the two new cases reported in this paper, it shows the complexity of the factors producing the purpuric eruption. The appearance of cutaneous hemorrhages is in no way a simple phenomenon and its mechanism is still unknown. The observations of Bedson has shown that the experimental induction of petechiae and hemorrhages sometimes requires the cooperation of a hemic and a vascular factor. Reilly, Rivallier, Compagnon, Laplane, and Du Buit,<sup>6</sup> later confirmed by Frumusan,<sup>3</sup> have demonstrated the role of the autonomous nervous system in animals in the production of some hemorrhagic lesions of the gastrointestinal tract. These very interesting experiments do not, however, afford a satisfactory explanation of the clinical observations concerning the apparition of purpura.

We hope that this paper will draw attention to this important problem, and that it will make experimenters and clinicians conscious of the inconstancy and variability of the vascular fragility test.

#### SUMMARY

Two unusual cases of purpura simplex raise the problem, as yet unsolved, of the pathogenesis of the purpuric eruption. Together with a previous observation of hemorrhagic purpura made by Roskam, they show the inconstancy and variability of the vascular fragility test.

#### ACKNOWLEDGMENT

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# THE VALUE AND THE LIMITATIONS OF THE COAGULATION TIME IN THE STUDY OF THE HEMORRHAGIC DISEASES

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THE DETERMINATION of the coagulation time of the blood is among the most empirical procedures routinely employed in the clinical laboratory, and is one most prone to be misinterpreted. In a critical evaluation of this test, one must consider first the mechanics of the procedure and second the physiological aspects, which require a translation of an *in vitro* observation into a probable *in vivo* behavior that is coordinated with other factors bringing about hemostasis. For this task a short historical survey is helpful since one can thereby acquire a knowledge of the evolution and development of the tests of coagulation time that are now in common use.

Although delayed coagulation of shed blood in various conditions was observed even in antiquity, there appears to have been no formal clinical test until 1878 when Vierordt<sup>1</sup> devised a procedure consisting of drawing a horse hair through blood in a capillary tube and observing first the point when fibrin threads adhered and again when the hair was free. In 1893 Wright<sup>2</sup> determined the coagulation time by filling capillary tubes with blood and noting the time when the contents could no longer be discharged by blowing. This investigator appears to have been the first to state specifically that the coagulation time of hemophilic blood was delayed. Brodie and Russell<sup>3</sup> three years later described a special instrument called a coagulometer in which a hanging drop of blood observed under a microscope is played upon by a current of air and the time determined for arresting the movement of erythrocytes. In 1898, Hayem<sup>4</sup> introduced the simple procedure of putting venous blood in a test tube and noting how much time was required before a sufficient clot was formed to permit tilting without a flow of blood. Fifteen years later Lee and White<sup>5</sup> employing the same principle devised a test which with minor modification has become the most widely used and most acceptable method for estimating the coagulation time. It is this test which is critically studied in this paper.

Several other tests should, however, be mentioned because they have in the past been employed extensively. Two of these methods were described in 1904. The first was Burker's<sup>6</sup> in which a fine glass rod is passed repeatedly through a drop of blood thereby catching the first strands of fibrin formed. The other was devised by Sabrazès<sup>7</sup> who filled capillary tubes with blood and at regular intervals of time broke off a short piece until a fibrin thread appeared between the severed sections. Fuld and Schlesinger,<sup>8</sup> in 1912, introduced another approach, blood was placed in a U-tube and the movement of a metal bead observed as the tube was gently tilted and the moment timed when the density of the clot fixed the bead. A modification of this method by Hedenius<sup>9</sup> is still widely used especially in the Scandinavian

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countries. For a detailed and complete review of this subject, Nygaard's monograph<sup>10</sup> should be consulted.

*Interpretation of the coagulation time* All the common procedures for determining the coagulation time are based on timing the interval between the removal of the blood from the vessel and the formation of sufficient fibrin to meet an arbitrarily designed end point. The objective of every test is to measure the intrinsic coagulative power of the blood. Obviously therefore, trustworthy results can be obtained only by performing the test under constant and rigidly controlled conditions and by excluding all outside agents that influence the coagulation reaction. Of the latter, tissue juice is by far the most important since it contains thromboplastin. It is therefore of utmost importance to exclude all traces from the specimen of blood used for determining the clotting time. Obviously, blood obtained by skin puncture (capillary blood) is utterly unsuitable since not only does it contain an appreciable amount of tissue fluid, but even more serious, the amount cannot be controlled. Thus Christie<sup>11</sup> demonstrated that there were variations in the clotting time of the different drops of blood collected from the same puncture, and Lee and White<sup>6</sup> cite the example of a hemophilic blood which had a coagulation time of 50 minutes for venous blood and only 5 minutes for capillary blood. It must be emphasized that the coagulation time of capillary blood, irrespective of the method used, is worthless and unreliable for clinical purposes. Even in taking blood by veni-puncture enough tissue thromboplastin may occasionally gain entrance to reduce significantly the coagulation time as Jaques and his co-workers<sup>12</sup> have recently demonstrated. It is not unusual to obtain a coagulation time of 8 to 10 minutes in a hemophilic subject, whose true coagulation time is 1 hour, merely by causing slight trauma in drawing the blood.

*Standardization of the coagulation time* There is a distinct need for a simple and uniform procedure. In the United States the Lee-White test is gradually replacing the other methods. This procedure is simple and yields as much and as accurate information as any test of coagulation so far devised, but unfortunately the test has not been rigidly standardized and at present there is no strict uniformity in the details of the procedure.

In order to devise a standard procedure, it is necessary to consider the more important factors that influence the coagulation of blood in a test tube. They are (1) temperature, (2) size of tube and (3) the nature of the surface of the tube.

*Temperature* Since the coagulation of blood involves chemical and enzymatic reactions, it is obvious that the temperature must significantly influence the speed of the process. This is clearly demonstrated by the results in table 1 in which the clotting times obtained at 22° C and 37° C are recorded. The marked effect on hemophilic blood is particularly noteworthy. Similar observations have been recorded by Patch and Stetson<sup>13</sup>. The need for adopting a constant temperature at which the determination is carried out is evident. Body temperature (37° C) seems most desirable since it is at that temperature that coagulation takes place physiologically. An ordinary vacuum bottle filled with water at 37° C serves as a handy water bath for carrying out the coagulation time test. The practice of performing the test at room temperature should be abandoned since the temperature range in many laboratories may be from 20° C to 30° C and this causes considerable difficulty and confusion in interpreting the clotting time and in deciding whether it is within normal limits.

*The size of test tube* The original Lee-White procedure specified the use of a Widal tube 8 mm in diameter, but test tubes of varying sizes are now employed. To show the effect of size of tube, the clotting times of bloods in test tubes with internal diameters of 8 and 11 mm were compared. Curiously, normal blood clots more rapidly in the smaller tube while hemophilic blood clotted considerably faster in the

larger test tube (table 1). A possible explanation is that the clotting time is influenced by both the area of the surface in contact with the blood and the surface of the blood exposed to air. In normal blood the disintegration of platelets is probably roughly proportional to the amount of foreign surface, and therefore the smaller the tube the greater the surface and the faster the disintegration of platelets. In hemophilic blood the platelets in contact with a foreign surface do not readily undergo lysis and therefore the contact of the blood with air has a more predominating effect. The influence of exposure to air is particularly well demonstrated by normal plasma deplateletized by high centrifugation. Coagulation begins at or near the air-plasma interface and travels downward.

Since the size of tube definitely influences the speed of coagulation, it becomes necessary to standardize by selecting a fixed size of tube. Since nearly every laboratory is well supplied with serological test tubes (13 x 100 mm) and since this is a convenient size both for handling and cleaning, it seems logical to select this tube for the test.

TABLE 1—*The Effect of Temperature, Size of Test Tube and Surface of Test Tube on the Coagulation Time*

		Coagulation time in minutes					
Subject	Type of test tube	Glass*	Glass*	Glass*	Lusteroid	Collodion coated	Silicone coated
	Internal diameter	11 mm	11 mm	8 mm	13 mm	11 mm	11 mm
	Temperature	22°C	37°C	37°C	37°C	37°C	37°C
Normal	I	13	6 $\frac{1}{2}$	4 $\frac{1}{2}$	10 $\frac{1}{2}$	26	47
	II	14 $\frac{1}{2}$	6 $\frac{1}{2}$	5	14	23	41
	III	12	8	4 $\frac{1}{2}$	12	29	52
	IV	10 $\frac{1}{2}$	6 $\frac{1}{2}$	4 $\frac{1}{2}$	10 $\frac{1}{2}$	27	43
	V	16	5 $\frac{1}{2}$	4	16 $\frac{1}{2}$	29	36
	VI	16	7	5	20	28 $\frac{1}{2}$	42
Hemophilic	I	121	65	90	360	140	450
	II	76	25	31	190	240	340

\* Pyrex

*Nature of the surface of the tube.* Blood clots fastest in glass as can be seen from the results in table 1. In a lusteroid (a synthetic plastic) tube, coagulation is definitely delayed, and in collodion coated tubes the retardation is even more marked. Silicone (Dry Film) coating, however, is the most effective artificial surface known for preserving the fluidity of blood.

A glass test tube is best suited for determining the coagulation time, since as much information is obtained as would be by employing any other type of tube, and it obviates the long waiting period which is always undesirable in a clinical laboratory.

*Recommended procedure for determining the coagulation time.* Blood is drawn by vein puncture preferably with a No. 22 needle, into a dry syringe. If the determination cannot be made immediately, a syringe coated with silicone (Dry Film)\* should be used, and the blood kept in the syringe until the operator is ready for the test. In drawing the blood the tourniquet should be applied just prior to the puncture. If blood is not obtained immediately and without trauma, another vein should be selected and a new puncture made. One cubic centimeter of blood is transferred into each of 2 scrupulously cleaned test tubes (Pyrex 100 x 13 mm). Since the test tubes are apt to vary slightly in size, only tubes having an internal diameter of 11 mm should be selected. The tubes are immediately placed in a water bath kept at 37°C. A vacuum bottle fitted with a cork having a hole in which the test tube can be inserted serves as a handy portable water bath. The tube is gently tilted every 30 seconds and the end point taken as the moment when on tilting no flow of blood is any longer observed. The normal range is 5 to 10 minutes with the majority of bloods clotting between 6 and 8 minutes.

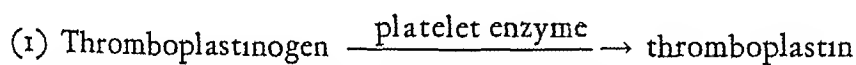
\* Manufactured by the General Electric Company and marketed as Dry Film No. 9987

It should be emphasized that the end point selected is arbitrary and does not mark the time of complete coagulation. Unconverted fibrinogen may still be demonstrated. A convenient way to measure incipient coagulation is to insert a glass rod coated with collodion into 1 cc. of blood and then withdraw it gently every 30 seconds. A fine thread of fibrin marks the beginning of coagulation. In normal blood, coagulation usually begins in  $3\frac{1}{2}$  to 4 minutes and is complete in 10 minutes, whereas in hemophilic blood coagulation may begin (to cite a specific observation) in 10 minutes but require 2 hours more before enough fibrin is formed for a solid clot. The tube with the glass rod should not be used for determining the final coagulation time.

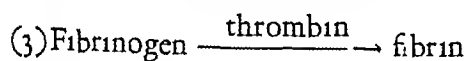
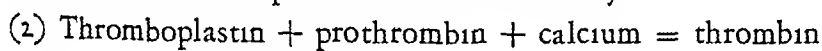
### A NEW THEORY OF BLOOD COAGULATION AND ITS BEARING ON THE COAGULATION TIME

As the result of recent studies,<sup>14</sup> evidence has been obtained to show that platelets do not furnish thromboplastin, but in their disintegration liberate an agent, probably an enzyme, that activates thromboplastin which occurs in the plasma as a precursor and for which the term, *thromboplastinogen*, has been proposed. It is probably identical with the antihemophilic globulin of Minot and Taylor and the prothrombokinin of Lenggenhager.

According to this new concept, the first step in coagulation can be expressed as follows



The activated thromboplastin reacts immediately



The first and third equations are enzymatic, whereas the second is stoichiometric. Thus, even a small number of platelets are sufficient to activate enough thromboplastin to furnish a quantity of thrombin that will coagulate blood within the normal period of time. Such a quantity of thrombin may, however, be entirely inadequate, as will be discussed later, to meet the hemostatic requirements. To understand the significance of the coagulation time, it should be remembered that normal human blood could clot in 12 seconds if it had an optimum amount of thromboplastin and that furthermore, the curve correlating the coagulation time and the concentration of thromboplastin is a hyperbola with both asymptotes zero. This explains why the shortening of a coagulation time from 1 hour to 5 minutes can be brought about by an extremely small quantity of thromboplastin. The amount of thrombin formed depends not on the coagulation time but on the quantity of prothrombin, thromboplastin, and calcium in the plasma and this can be looked upon as the key to a better understanding of several important hemorrhagic diseases which will be considered in this presentation.

The coagulation time may be prolonged in four well known diseases or conditions: hemophilia, hypoprothrombinemia, afibrinogenemia and heparinemia. It is possible that a delayed coagulation may occur in other conditions, but these have not been studied sufficiently to permit critical analysis. Hypercoagulability remains a vague and as yet meaningless term.



## THE COAGULATION TIME IN HEMORRHAGIC DISEASES

*Hemophilia* With the exception of complete incoagulability of the blood as encountered in afibrinogenemia, the most prolonged coagulation times are observed in hemophilia. A coagulation time of one hour is not unusual, but a time of two hours or more is rather infrequent, provided the test is done carefully and at 37° C. It has been found in recent studies that the coagulation time of a hemophilic may be surprisingly constant for a relatively long period of time. Thus, the coagulation time of one subject has remained about 55 minutes with few exceptions during the past 18 months. Although it has been brought to normal several times with plasma transfusion it has always promptly returned to this rather fixed value. The same constancy has also been found in other hemophilics, but in no instance has the period been long enough to be significant.

To understand the coagulation time in hemophilia, it is necessary to understand the basic defect in this disease. In a recent study<sup>14</sup> it has been found that hemophilic blood is almost completely devoid of thromboplastinogen, and even after all the fibrinogen has coagulated, no demonstrable consumption of prothrombin has occurred.\* All the coagulation is due therefore to a minute quantity of thrombin which is formed and which, because it is an enzyme, can convert all of the fibrinogen to fibrin in a relatively short time. The minuteness of the quantity of thromboplastin which can bring about a normal coagulation time is clearly demonstrated by the following experiment.

A stock extract of thromboplastin prepared by mixing 0.2 gm. of dehydrated rabbit brain in 5 cc. saline, was diluted 1 to 1000. On adding 0.1 cc. of this diluted thromboplastin to 1 cc. of a hemophilic blood which had a coagulation time of 2 hours and 15 minutes, the time was reduced to 5 minutes. This 0.1 cc. of thromboplastin contained only 2.5 gammas of solid material, of which a large fraction was inert. Obviously, the amount of thrombin formed must have been extremely small, yet it coagulated the blood in 5 minutes. The conversion of prothrombin, however, was so small that it could not be demonstrated.

From the results observed in hemophilia and in hypoprothrombinemia it seems definite that hemostasis is not dependent on the clotting time but on the quantity of thrombin supplied during the clotting process. In hemophilia little thrombin is formed since the plasma lacks the thromboplastin precursor. Even if the plasma contains enough thromboplastinogen to cause a normal coagulation time, it may not be sufficient to supply enough thrombin for the hemostatic needs. This explains why a normal coagulation time may be found in known hemophilics suffering from repeated hemorrhages. In a limited number of such patients, the senior author could demonstrate no consumption of prothrombin after coagulation had been completed. Such patients are a problem to the surgeons since the normal coagulation may create a false sense of security. Furthermore, not every measure which reduces the coagulation time of a hemophilic is necessarily effective in controlling hemorrhage. Just as the effectiveness of vitamin K cannot be established

\* A new procedure named the prothrombin consumption test has been developed. It consists in determining (by the senior author's method) the prothrombin remaining in the serum 1, 3, and 24 hours after the blood has clotted.

by the coagulation time but only by the decrease in the prothrombin time, so the assay of any antihemophilic agent cannot be made with an absolute degree of certainty by the coagulation time, but will probably require the measurement of the prothrombin consumption

The coagulation time is obviously of limited value in hemophilia either in the diagnosis or in the treatment. A prolonged value is suggestive of hemophilia provided other causes are ruled out. A normal coagulation time does not exclude a diagnosis of hemophilia. A history of bleeding and a markedly poor consumption of prothrombin during coagulation appears to be much more reliable evidence on which to base a diagnosis.

The coagulation time, is, however, of some practical and theoretical value. A hemophilic with a coagulation time that is nearly normal usually has mild attacks of bleeding and only encounters serious trouble when relatively large vessels are damaged. The severity of the bleeding tendency appears to be relatively independent of the coagulation time when the value of the latter exceeds 15 to 20 minutes. In three hemophiliacs having average coagulation times of 25, 55 and 120 minutes respectively, the frequency and severity of the bleeding episodes during a period of observation of 6 months, was roughly the same. Theoretically, the coagulation time is of value since it offers the only means to grade the severity of the hemophilic defect. Thus the difference in availability of thromboplastin between the three hemophiliacs mentioned is so small that no other test, including the prothrombin consumption, can detect the difference.

The coagulation time has, it should be mentioned, served not only in establishing the presence in plasma of an antihemophilic agent, but has enabled Minot, Taylor and their associates<sup>16</sup> to concentrate it. They wisely depended not so much on a transient lowering of the coagulation time but on a sustained normal value.

*Hypoprothrombinemia* Prior to the advent of vitamin K, it was very puzzling to the surgeon why the jaundiced patient bled postoperatively in spite of a normal coagulation time. The senior author, on the basis of his early studies on vitamin K, concluded that the hemorrhagic danger level was indicated by a prothrombin time of about 25 seconds, which corresponds in man to a prothrombin activity of 20 per cent of normal. At this level the coagulation time is so little increased that unless the test is done with great care it escapes detection, since it is still well within the normal range. In fact, it has been found<sup>16</sup> that in dogs an increase of the prothrombin time from the normal of 6 seconds to 60 seconds is accompanied by a change of the coagulation time only from  $3\frac{1}{2}$  to  $5\frac{1}{2}$  minutes, i.e., an average increase of only 2 minutes. Even with extremely low concentrations of prothrombin, the coagulation time is rarely as prolonged as in moderately severe hemophilia. At very low levels, the prothrombin time and the coagulation time tend to become identical. Thus, on reducing the prothrombin in a dog with dicumarol until the prothrombin time was 20 minutes, a coagulation time of 19 minutes and a clotting time for recalcified plasma of 30 minutes was obtained. The likely reason for such a result is that the limiting factor is prothrombin and that under such circumstances the thromboplastin of the plasma is adequate, and therefore additional amounts of the latter have no further effect.

Early in the work on toxic sweet clover poisoning, one of us<sup>17</sup> discovered that a heart puncture in a rabbit with a reduced prothrombin caused fatal hemotopericardium. This serves, therefore, as a useful means to study hemostatic effectiveness, and

TABLE 2.—*The Relationship of the Coagulation Time, Clotting Time of Recalcified Plasma, and Prothrombin Time to the Hemostatic Breakdown*

Day	Rabbit	Prothrombin time	Coagulation time (Lee White†)	Clotting time of recalcified plasma	Effect of heart puncture‡
		sec	min	sec	
	1*	6	13½	185	
	2	6	13½	165	
	3	6	14	175	
	4	6	12½	165	
	5	6	14	180	
	6	6	12½	165	
1	1	6	13	170	
	2	11	14	240	
	3	12	15½	255	
	4	9	12½	210	
	5	11	15	225	
	6	10½	14	225	
2	1	6	14	180	
	2	25	15	450	Fatal hemotopericardium
	3	19½	16½	405	
	4	13½	14	255	
	5	14½	16½	255	Fatal hemotopericardium
	6	27	19	495	
3	1	6	13	175	
	3	24½	18½	450	Fatal hemotopericardium
	4	18	15½	300	
	5	25	17½	420	Fatal hemotopericardium
4	1	6½	13	180	
	4	24½	16½	435	Fatal hemotopericardium

\* Control the other five rabbits were given 2 mg per kg of body weight of dicumarol daily by stomach tube

† Blood was obtained from the median artery of the ear with a silicone coated syringe, which probably accounts for the long normal coagulation time

‡ The heart punctures were made with a No. 21 needle and always approximately in the same position

In table 2 a correlation is made between the prothrombin time, coagulation time, and the hemostatic breakdown. A study of these results clearly shows that when the prothrombin time is less than 19 seconds the animals' blood could prevent cardiac bleeding. With a prothrombin time of 24 seconds or more, hemotopericardium

invariably occurred. While there was a slight increase in coagulation time, no clear cut relation between it and fatal hemorrhage could be found. Interestingly, one of us has observed a series of cases with congenital hypoprothrombinemia and has found that those with a prothrombin time of 16 seconds showed no hemorrhagic tendency, two cases with a prothrombin time of 19 seconds showed a distinct bleeding tendency, and one case with a value of 30 seconds showed a very severe hemorrhagic condition.

Obviously the coagulation time is of little or no value in the study of hypoprothrombinemia. It cannot be used for controlling dicumarol therapy. Again basically the fact is brought out that hemostasis depends on the amount of thrombin formed, and when the prothrombin is reduced to about 20 per cent insufficient thrombin is furnished for stanching.

*Afibrinogenemia* When total incoagulability of the blood is found, afibrinogenemia should be suspected and a qualitative test for fibrinogen made. Recently, Pinniger and Prunty<sup>15</sup> demonstrated experimentally that the prothrombin time remained approximately normal in the blood of their patient until the fibrinogen fell below 50 mg per 100 cc of plasma, and that the Lee-White coagulation was 5 minutes when the fibrinogen concentration was as low as 30 mg. It is obvious that the coagulation time has little practical value in this hemorrhagic condition except in the initial detection of a coagulation defect.

*Heparinemia* Animals, particularly dogs subjected to peptone or anaphylactic shock, respond by an outpouring of histamine and heparin into the blood, and by a marked thrombocytopenia. The resulting heparinemia may be so great that the blood is rendered incoagulable. In man, the appearance of heparin in the blood has not been unequivocally demonstrated although there is a good probability that it can occur. The increase of the coagulation time is not necessarily proportional to the concentration of heparin. The latter can be much more accurately determined by titration with progressive dilutions of a standard thrombin solution.<sup>19</sup>

The therapeutic use of heparin in the prophylaxis of thrombosis is successfully controlled by the coagulation time, but this is entirely on an empirical basis, since it has not been accurately determined how much heparin is needed for this purpose. It is probable that the effective action of heparin consists in neutralizing thrombin, and thus reduces the effective quantity of the latter.

From the foregoing discussion, it becomes clear that the coagulation time has limited value in the study of the known hemorrhagic diseases. It has, however, an important function in the possible discovery of new hemorrhagic diseases. On finding a prolonged coagulation, a concise diagnosis can be made only by specific tests such as the prothrombin time, and the prothrombin consumption test. A little over a decade or two ago, hemophilia was the waste basket for nearly all hemorrhagic diseases characterized by a coagulation defect. Since then hypoprothrombinemia, afibrinogenemia, and heparinemia have been recognized as separate entities. It is highly probable that other hemorrhagic conditions having a prolonged coagulation time exist but thus far have not been recognized and defined because of a lack of suitable methods of study.

## SUMMARY

The coagulation time is a measurement of the intrinsic power of the blood to convert fibrinogen to fibrin. It is an empirical test no matter how performed, and therefore in order to be reliable requires that the test be done on venous blood under strictly controlled conditions. A recommended procedure is outlined in detail.

The coagulation time is prolonged in hemophilia, hypoprothrombinemia, afibrinogenemia and heparinemia. In hemophilia, the coagulation time theoretically is a measure of the severity of the disease but practically is of limited value since the coagulation time may be within normal limits in some patients, the prothrombin consumed in the coagulation of hemophilic blood is therefore a better guide for diagnosis. The coagulation time in hypoprothrombinemia is relatively little prolonged until a drastic reduction occurs. The test is therefore of no value for establishing a hemorrhagic condition in hypoprothrombinemia. In afibrinogenemia the blood is incoagulable. A small amount of fibrinogen restores the coagulation time to normal.

The presence of heparin increases the coagulation time. The test is therefore useful in controlling the therapeutic action of this drug.

The senior author, in making a survey of the literature on hemorrhagic diseases in preparation of his monograph, was impressed by the significant and diverse contributions which Dr. George R. Minot made to this field of medicine. We feel honored to contribute this study to the collection of papers offered as a fitting tribute to Dr. Minot, who has so successfully and productively combined science and clinical medicine.

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# THE ACTIVATION OF PROTHROMBIN, WITH SPECIAL REFERENCE TO "THROMBOPLASTIC ENZYME" (TRYPTASE)

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THE BASIC mechanisms whereby plasma prothrombin is converted into active thrombin, in order to coagulate fibrinogen solution to fibrin clot, can be shown by an experimental analysis of the isolated phases of clotting in vitro.<sup>23</sup> The theoretic goal would be to isolate *prothrombin* in a chemically pure state and subject it to test reactions in order to learn the various factors which participate in its activation to thrombin. In actual practice, however, the criteria of purity are difficult to define, particularly in view of the fact that mere traces of certain factors<sup>14-20</sup> suffice to cause a considerable degree of activation if operating over a long enough period. Such "trace impurities" quite fail to show up in the usual criteria, e g , constant solubility,<sup>61</sup> unicomponent electrophoretic pattern and isoelectric point,<sup>62</sup> and other data valid in protein chemistry.<sup>7</sup> It is most necessary, therefore, to supplement the examination of "purified" clotting agents with a series of sensitive tests for the presence and modes of action of these impurity factors.

Seegers and colleagues<sup>61</sup> have carried the difficult problem of prothrombin purification to a point which is very satisfactory from the viewpoints of biochemistry and physiologic potency. Through the courtesy of Dr. Seegers, we have been supplied with a number of these prothrombin preparations, the experimental analysis of which provides the bulk of data herewith reported. It must be emphasized that the results of these tests in no wise detract from the signal advance which these prothrombins represent in terms of potency and of freedom from all but traces of contaminants.

## I REAGENTS

1. *Borate buffer* (buff.) 45 vol. 2.5 per cent  $H_3BO_3$ , 45 vol. 0.5 per cent NaCl, 10 vol. 4 per cent  $Na_2B_4O_7 \cdot 10 H_2O$  (a simplification of the formula given by Burdon<sup>23</sup>) is used as solvent and diluent (e g , to constant volumes) throughout. It maintains a constant pH of 7.7 and largely controls the ionic strength of all our solutions. Its mild bacteriostatic properties are valuable in permitting the prolonged keeping and observation of the protein solutions, e g , thrombin mixtures, for several weeks and fibrinogen for many days at room temperature, and fibrinolytic tests (and controls) for similar periods at 37 C, with only a very occasional contamination by molds and bacteria (e g , T<sub>2</sub><sup>\*</sup>, table 8), even without meticulous sterilization of glassware, etc., and more than simple corking of tubes to exclude dust and prevent evaporation.

2. *Fibrinogens* (a) *Bovine plasma Fraction-I* (B F), Armour Lab., courtesy of Dr. J. B. Lesh, has proved to be a highly satisfactory fibrinogen preparation for quantitative clotting-time studies. An 0.5 per cent solution in borate buffer requires filtration of only a trace of insoluble fibrin-like material. Solutions should be kept at cool room temperature (20 C), not in the icebox, since the protein solubility is reduced.

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by borate at low temperatures. The insignificant traces of thrombin which form in solution may cause a slight fibrinous flocculation in four to six days (average). Owing to the broad zone of optimal fibrinogen concentration for clotting (Table 2), this trace of fibrin may be filtered off and the solution used for a day or two longer. However, this is not recommended in tests of the highest accuracy and it is unnecessary, since a new batch of B F solution can be relied on to give identical clotting-time values with similar thrombin solutions. Rigid tests, with added  $\text{CaCl}_2$  and thromboplastin, show a trace of prothrombin, but this requires many hours to activate and then produces a mere trace of clot. By no criteria can these facts be regarded as in any way interfering with the data to be presented. Armour's B F is significantly free from trypsin, tryptogen, and enzyme inhibitors (v. infra). It is stated to yield about 60 per cent of fibrinogen (clottable N).

(b) *Human plasma Fraction-I* (H F) or antihemophilic globulin (crude,<sup>45</sup> Harvard Laboratory, courtesy Drs. E. J. Cohn, J. T. Edsall, G. R. Minot and colleagues), has a similar (60 per cent) fibrinogen content, a somewhat (but not unduly) greater contamination with fibrinous and thrombic impurities, and a trace of active protease (trypsin), together with significant amounts of its precursor (tryptogen),

TABLE 1—Data on Prothrombin Preparations

Potency and stages of purification according to Seegers, W. H., Loomis, E. C., and Vandenberg, J. M. *Arch. Biochem.* 6: 85, 1945.

Description		Stage purified	Thrombic potency (specific activity)		Trace impurities (see text)			
Exp. tag	Lot No.		u./mg. tyrosine N	u./cc. 0.2% soln	Thrombin	Ca	Phospholipid	Trypsin
A	F101	Product 4	—	—	+	+	+	tr
B	S460904	*	15200	—	+	tr	tr	o
C	S460315	*	5800	—	tr	?	tr	o
D	S460404	*	9400	—	tr	tr	+	o
E	S461104	*	12600	2000	+	tr	+	o
F	S460923	*	13700	1400	+	tr	+	tr
G	S461219	Product 5	—	1320	tr	+	+	+
H	S470421	*	9900	—	tr	tr	tr	o

\* Step 7 carried through ammonium sulfate and isoelectric precipitations, frozen and dried (See *Arch. Biochem.* 6: 90, 1945).

which is best activated with Garner and Tillet's (1934) streptococcal agent now renamed streptokinase.<sup>6</sup> In the present studies, H F is used only as a source of plasma protease (q. v.).

3. *Prothrombins* The data reported in this paper are obtained by the use of several prothrombin preparations purified to various stages of the method of Seegers, Loomis, and Vandenberg.<sup>61</sup> PRO A was prepared, by ourselves, from citrated dog plasma, while the others, bovine PRO B-PRO H, were supplied by Dr. Seegers. A summary of data on these materials is appended in table 1 and their coagulant properties are discussed fully in the text. Brief allusion is also made to older Howell-type<sup>15</sup> crude prothrombin, precipitated by acetone from heat defibrinated (56 C) dog plasma, and dried on filter papers.

4. *Activators of prothrombin* (a) *Calcium salt* (Ca) M/10  $\text{CaCl}_2$  is prepared from stock M/1 (11.1 per cent)  $\text{CaCl}_2$  by dilution, preferably with borate buffer.

(b) *Cephalin* (ceph) the purified phospholipid isolated from brain<sup>16</sup> is briefly alluded to in the text.

(c) *Thrombokinase or thromboplastin* (tpln) the preparations repeatedly used in these studies, are filtered (glass wool) borate buffer suspensions of several commercial thromboplastins, designed for use in plasma prothrombin assay tests, e. g., tpln A Squibb's (rabbit brain), tpln B Difco's (rabbit brain), tpln C Sharp and Dohme's (horse brain). On a few occasions we used frozen dog brain (tpln D). The universal finding of a trace of proteolytic enzyme in all these crude thromboplastins is noteworthy.

(d) *Thromboplastic enzyme* see *Proteases*.



5 *Inhibitors of Prothrombin Activation* (a) *Citrate* (citr) 1/10 vol 4 per cent trisod citrate (hydrated) is optimal for (1) plasma preservation and (2) inhibition of 'spontaneous' activation of prothrombin solutions (see text)

(b) M/1 oxalate ( $K_2C_2O_4$ ) or citrate ( $Na_3C_6H_5O_7$ ), added to an equal vol of thrombic mixture, are best for progressive inactivation of thrombin-<sup>51</sup> intermediary (see text)

(c) *Heparin* (hep) 1 to dil (borate buffer) of Lederle's Sod heparinate (1 per cent) gives a solution containing 100 Toronto units per cc, which does not alter the pH of the buffer

(d) *Trypsin inhibitors* see *Proteases*

6 *Thrombin* For following fibrinogenolysis (e.g., table 13A) or clot-lysis, when the protease is to be studied in the fibrin, it is necessary to use an enzyme-free thrombin. A convenient preparation for this purpose is a 1 per cent solution of lyophilized rabbit hemostatic globulin<sup>52</sup> (h.g.), Lederle Laboratory, courtesy Dr I. A. Parfentjev. Some of the products, especially those supplied in solution for topical hemostasis,<sup>53</sup> are less suitable, however. Other commercial thrombins, viz (TU) Upjohn's, courtesy Dr J. T. Correll, and (TBD) Parke Davis, courtesy Dr E. A. Sharp,<sup>54</sup> are alluded to in table 20.

TABLE 2.—Clotting-times in Relation to Concentration of Fibrinogen

C t (in seconds), at 25 C, for 0.5 cc fibrinogen (B F-strengths\* cited) + 0.5 cc thrombin (h.g., 1%)—see *Reagents*

Conc (%)*	20	10	0.5	0.2	0.1	0.05	0.02	0.01
Clotting-time (in seconds)	6	5	5	6	7	10	47	115

\* Grams of original material (B F 60 per cent clottable fibrinogen) per 100 cc

TABLE 3.—Clotting-times in Relation to Concentration and Age of Thrombin Solution

C t (in seconds), at 20 C, for 0.5 cc B F (0.5%) + 0.25 cc thrombin (T)\*

Rel T conc (%)	100	50	25	10	5	1	0.5
	(Time in seconds)						
Aged ¼ min	4	8	11	22	41	84	117
30 min	4	8	11	22	48	104	194
60 min	4	8	11½	24	54	130	205

\* T (100%) = 3-day old mixture of 4 cc PRO G (0.4%) + 5 cc buff + 0.5 cc tpn D + 0.5 cc  $CaCl_2$  (M/10)—see T<sub>6</sub>, table 8

7 *Proteases* (a) *Active enzyme* (1) *Pancreatic trypsin* (a) *crystalline trypsin*, from pancreas, courtesy Dr M. Kunitz<sup>44</sup> (Rockefeller Institute, Princeton) is desirable for such special purposes as distinguishing effects from those of chymotrypsin. However, for most practical purposes, e.g. 'standard' for routine protease assay by the fibrinogenolytic method,<sup>21</sup> (b) *commercial trypsin* (Fairchild Bros and Foster), will suffice. This trypsin (tryp) in the form of a 2 per cent extract made with equal vols of glycerol and borate buffer (cf Burdon<sup>3</sup>), filtered, and stored in the ice-box, is the stock solution from which standard dilutions are freshly made (with borate buffer) immediately before use. One trypsin UNIT is the fibrinogenolytic potency (for 0.25 per cent B F) of 0.01 mg (per cc) of our standard trypsin. Lysis tests are conducted in the warm room (37 C) or water-bath (39 C).

(2) *Trypsin preparations* (a) The Harvard human plasma fraction (III-3), supplied through the courtesy of Dr J. T. Edsall,<sup>55</sup> has considerable proteolytic properties, together with much thrombic activity (see text), (b) *Dog plasma trypsin* (tryp D) is prepared by a method we have not yet perfected (particularly from the point of view of stability of the enzyme preparation) but the product may be characterized as able to give complete fibrinogenolysis in 3 to 5 minutes and clot-lysis in 9 to 10 minutes at room temperature, when using 0.5 per cent B F, or fibrin clot therefrom, as substrate, (c) *Crystalline*

*human serum albumin* (H S A) and *crystalline bovine serum albumin* (B S A), Harvard Laboratory, courtesy Dr J T Edsall,<sup>27</sup> are found to be contaminated with a trace of active trypsin

(b) *Preparations containing enzyme-precursor (tryptogen)* Many plasma protein fractions contain, in addition to traces of active trypsin, varying amounts of tryptogen (enzyme-precursor), which can be activated by shaking with chloroform,<sup>28</sup> or, better, by using streptokinase<sup>6</sup> (v infra)

(1) *Human plasma Fraction-I* (H F), v supra, and the more refined (85-90 per cent clottable) but less stable (2) *human fibrinogen* (H Fb<sup>33</sup>), products of the Harvard Laboratory (courtesy Dr E J Cohn and colleagues), are representative of this group, and so are (3) various (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-pptd fibrinogens which we prepare from dog plasma (D Fb) On the other hand, Armour Laboratory bovine fibrinogen and the more satisfactory fibrinogen precipitated from bovine Fraction-I (B F) by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> are as enzyme-free as the cruder fraction<sup>33</sup>

TABLE 4—Effect of Keeping Prothrombin Solution, with Reference to Activation and Thrombin Yields, with Various Activators

A *Activation series* C t (in seconds) at 25°C, pH = 7.7 (borate buff) for 0.5 cc B F (0.5%) + 0.25 cc T

T 5 cc vol, containing 4 cc PRO B (0.2%) + 0.25 cc M/10 CaCl<sub>2</sub> + 0.25 cc thromboplastic agent

T	Date	Activator	Incubation Period room temperature										Clot lysis (37 C)
			1 m	10 m	30 m	1 hr	2 hr	6 hr	1 d <sub>y</sub>	2 d <sub>y</sub>	4 d <sub>y</sub>	7 d <sub>y</sub>	
			seconds										
1	10-8-46	Ca only	112	87	66	48	38	16	9	7	3	3	0 (10 day)
2	"	Ca + t <sub>pln</sub> A	68	14	6	5	3	3	3	3	3	3	6-7 day
3	12-9-46	Ca + t <sub>pln</sub> D	20	12	8	4	3	3	3	3	3	3	not tested
4	10-8-46	Ca + tryp (4-u)	16	7	5	5	4½	6	19	47	100	—	3 day

#### B Thrombin dilution series

10-8-46 Undil T<sub>2</sub>, 2½ hrs old = 1 1, 0.5 cc B F (0.5%) + 0.25 cc T<sub>2</sub> dilutions

Rel T conc	1 1	1 2	1 4	1 8	1 16	1 32	1 64	1 128	1 256
Clotting-time, in seconds	3	6½	9	18	25	39	61	90	230

(c) *Protease activators* The streptokinase (strep), long miscalled streptococcal fibrinolysin, used in the present studies, is a 1 per cent extract (in borate buffer) of a potent preparation made by the Tillett and Garner method<sup>37</sup>

(d) *Trypsin inhibitors* Through the courtesy of Dr M Kunitz (Rockefeller Institute, Princeton), we received some crystalline pancreatic trypsin-inhibitor (T I<sup>32</sup>) and crystalline soybean trypsin-inhibitor (S B I<sup>44</sup>), the highly significant trypsin- and thromboplastin-inhibiting properties of which are discussed in the text

## II QUANTITATIVE METHODS

Owing to lack of methods for direct chemical analysis in dealing with such complex proteins as fibrinogen and prothrombin (or thrombin), reliable assay methods necessarily involve the whole coagulation reaction *Clotting-time* (c t), measured under carefully standardized conditions,<sup>15</sup> is the best and only currently practical solution to the problem of quantitative estimation of thrombin (*activated* prothrombin)

*Experimental conditions* <sup>63</sup> Being colloidal reactions,<sup>26</sup> the clotting processes are influenced by (1) temperature, (2) pH, (3) salt content, (4) concentration (dilution) of specific factors (v. infra), and (5) adsorption and related colloidal phenomena <sup>27</sup> In the last category are (a) effect of wettable surfaces, e. g., the well-known ability of blood to clot quicker in glass than in paraffined,<sup>36</sup> plastic,<sup>68</sup> or silicone treated<sup>41</sup> tubes, and (b) the clot-riding (second phase) or fibrinoplastic effects of a wide variety of nonspecific colloids, e. g. kaolin,<sup>56</sup> gum acacia,<sup>63</sup> salmine,<sup>18</sup> etc <sup>56</sup> The first step, therefore, is to standardize these experimental conditions

*Fibrinogen concentration and clotting-time* Table 2 shows the varying clotting-times when a given thrombin solution (1 per cent h. g.) is added to an equal volume (0.5 cc.) of a series of fibrinogen (B. F.) dilutions. Confirmatory of many older data,<sup>70</sup> there is found a broad optimum which, for this and similar fibrinogens,

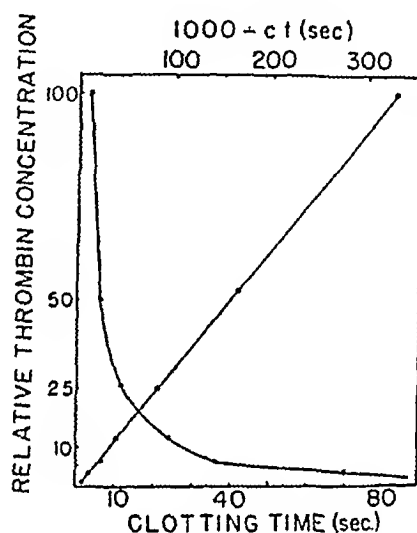


FIG. 1. CLOTTING-TIMES AND RELATIVE THROMBIN CONCENTRATION (PERCENTAGE) THE INVERSE LAW (ref. Am. J. Med. 3, 67, 1947)

Clotting-times (sec.) and  $1000 - ct$  (sec.) of 1 cc. fibrinogen (0.5% B. F.) + 0.25 cc. thrombin (T)  
Temp. 25° C., pH = 7.7 (borate buffer)

T = 2-day old mixture of 8 cc. PRO. C (0.2%) + 1 cc. buff. + 0.5 cc. tpin. A (0.25%) + 0.5 cc. M/10  $\text{CaCl}_2$

lies in the zone of concentrations between 1.0 and 0.25 per cent. A stable thrombin repeatedly gives identical  $ct$  values with a recommended 0.5 per cent B. F. solution every time this is made from the lyophile-dried plasma fraction.

*Thrombin concentration and clotting-time* Figure 1 shows the progressively shorter clotting-times when a series of thrombin dilutions (0.25 cc. vol.) are tested on a given fibrinogen solution (1 cc. 0.5 per cent B. F.) at pH = 7.7 (borate buffer) and room temperature (25° C.). The reciprocals of the clotting-times ( $1000 - ct$ , sec.), in this particular experiment, give a linear plot, illustrative of the so-called "inverse law."<sup>38</sup> In our experience, this law is of limited application in a restricted range of thrombin concentrations and under experimental conditions which it is not possible to define. It does not require rigid mathematical formulation, however, to grasp the fundamental fact that, under standard test conditions, a shorter clotting-time means more thrombin. Thrombin dilution series are given in a number of the following tables to indicate at least the order of magnitude of

the relative amounts of thrombin corresponding to the various clotting-times. The reservations with which we regard any clotting-test system in which the over-all *dilution* of the thrombin is changed led us to make the experiments of table 3.

*Thrombin instability during dilution experiments.* After complete activation of purified thrombins (see tables), the reproducibility of the clotting-time values in test after test often over a period of several weeks, is (to anyone who has experienced difficulties in keeping the older types of unstable thrombins) truly amazing. Yet any considerable dilution of these excellent thrombins at once introduces a factor of instability. This is frequently apparent in less than a hour at room temperature and more rapidly if the temperature is increased.<sup>15</sup> Table 3 shows thrombin dilution data first obtained immediately ( $\frac{1}{4}$  min) after making the dilutions and subsequently repeated, on the same solutions, after  $\frac{1}{2}$  hr and 1 hr, respectively, at 20 C. The stronger thrombins show no detectible change but the higher dilutions weaken progressively.

This phenomenon is encountered repeatedly with every type of thrombin studied, including the purest preparations (free from protease, for instance). Moreover, any destructive impurity would be in highest concentration and presumably most active in the stronger solutions. It makes no difference whether buffer or distilled water is used as the diluent. There is no turbidization to suggest denaturation. It can only be surmised that much remains to be learned about the way in which coagulant potency is related to the colloidal structure of the thrombin protein. Over-all *dilution* is undoubtedly a factor of considerable implication in the clotting system.<sup>16</sup> Not knowing the extent of these implications we believe it safest to insist upon always using a definite amount of prothrombin in a *constant volume* of thrombic- and clotting mixtures, since this approximates most closely the natural clotting conditions.

*Prothrombin activation data*, presented (1) in many tables (4-16) as actual clotting-times (c t), (2) (table 17) as computed thrombin "percentages" (rel T conc), or (3) (figs 2, 3), graphically ("activation curves"), in the course of these studies, are obtained as follows:

A given amount of prothrombin (*fixed* for each series of comparative experiments) is mixed with any activators (and inhibitors) it is desired to study, and the "thrombic mixture" (T) made up to *constant volume* with buffer solution. Actually the most potent activator, usually Ca, is added last and the *incubation period* (i t) recorded from this moment. At various periods, a measured sample (usually 0.25 cc) is removed from the thrombic mixture and added to a labeled 12 mm diameter Wassermann-tube containing the test fibrinogen (usually 0.5 cc 0.5 per cent B F) gently mixed and agitated, and the *clotting-time* (c t) noted with a stop-watch from the moment of mixing to the first appearance of definite fibrin threads.

The *end-point*, in our water-clear solutions, is sharp enough for an accuracy of 5 to 10 per cent, which is often but a fraction of a second. We ignore the earlier point of incipient turbidity and do not trust the later point of solidity (invertibility of tube), since this (a) is read with less accuracy, (b) involves some nonsignificant variables (e g diameter of tube) and (c) is often incomplete in the case of extremely weak thrombins.

Since there is only a minor ( $\times 3$ ) dilution factor to check continuing thrombin formation between adding the fibrinogen and the onset of clotting, theoretical objection may be raised on this point. That this is of little practical significance, however, follows from the fact that the most significant clotting-

times are measurable in a matter of seconds after a long (often hours or days) period of activation. Moreover, it tends to enhance the differences between a long clotting-time (due to slow rate of thrombin formation) and a short c t (due to rapid approach toward complete activation). A larger dilution factor, e g., 0.1 cc T + 1.0 cc B F has been tried on a number of occasions without significant benefit. Indeed, it is less suitable for use with very weak thrombins and the volumes are less conveniently measured and pipetted.

We have a number of objections to the use of oxalate or citrate in the fibrinogen,<sup>9</sup> e g., (1) continued thrombin formation is still possible, especially in the presence of protease (see thromboplastic enzyme), (2) with unduly large amounts of anticoagulant (fig. 2), there may be reversion of incompletely formed thrombin to prothrombin (see section on calcium and thrombin- "intermediary"), (3) there is certainly some second-phase inhibitory effect which may be excessive with very weak thrombins (compare  $T_1$  and  $T_2$ , table 8), (4) the c t end-point is less sharp (a) in the case of oxalate, because of opacity due to  $\text{CaC}_2\text{O}_4$  (whenever calcium is present), (b) in the case of citrate, because of undue translucency at the alkaline pH (7.7) of our borate buffer, although 0.1 to 0.4 per cent citration is permissible, at least with c t < 30 sec., without significant clot-timing interference.

It is to be emphasized that the significant differences between the activation (and other) data are readily appreciated by simple inspection.

*Second phase controls.* Whenever any question arises of possible clotting-time modifications due to a reagent having some effect on the *second-phase* (thrombin-fibrinogen interaction), we always run suitable controls, with several thrombins or thrombin dilutions. Examples will be noted in the tables.

TABLE 5—*Stability of Prothrombin in Presence of (Eram) Thromboplastin*

X = 20 cc PRO D (0.2%) + 20 cc tpln A (0.25%), at room temperature

T = 4 cc X (aged as indicated) + 0.75 cc buffer + 0.25 cc C<sub>1</sub>, activated at room temp for periods cited (1 t). Clotting-times (c t), in seconds for 0.25 cc T + 0.5 cc B F (1%)

T	age \	1 m	10 m	20 m	30 m	1 hr	3 hr	4 hr	18 hr (1 t)
		time in seconds							
1	20 sec	334	29	9	6	4	3	3	3
2	24 hr	568	85	30	15	7	3	3	3
3	48 hr	640	126	39	19	8	5	4	3
4	72 hr	727	155	52	24	10	5½	4	3
5	Control*	114	6	4	3½	3	3	3	3

\* Control 2 cc PRO D (72 hrs older) + 2 cc fresh tpln A (0.25%) + 0.75 buffer + 0.25 Ca.

N B The initial prothrombin solution (in X) had been prepared 6 days previously and stored in ice-box. Unfiltered tpln A was used in  $T_5$ .

### III EXPERIMENTAL DATA

*"Stability" of prothrombin in solution.* The tests of table 4 were made on a 0.2 per cent solution, in borate buffer, of PRO B, which, according to the stated (Seegers) potency of 15,200 thrombin "units" per mg tyrosine N (after activation) is one of the purest preparations. Initial tests, about 2 hours after making the solution, show a trace of active thrombin estimated to be < 0.5 per cent of the total potential thrombin yield, according to c t data in the accompanying *dilution series* (B).

*Activation tests (A)* were made initially on Oct. 8, 1946, with ( $T_1$ ) Ca, alone, and ( $T_2$ ) Ca + tpln A (0.1 per cent). The latter series was repeated ( $T_3$ ) two

months later, on Dec 9, with another thromboplastin (tpln D), the prothrombin solution having been kept in the ice-box and now showing considerable increase in thrombin content. Nevertheless, it is apparent from the data that there is still much prothrombin requiring activation. Although the activation is much slower in  $T_1$  (Ca, alone), it finally (4 days) reaches a stable optimum of thrombic potency which, as measured by the 3" c t, is identical with that in the other two series. While, therefore, the prothrombin solution is "unstable" in the sense that it slowly changes to thrombin and the more rapidly, the more favorable the conditions for activation, yet it is amazingly stable in the sense of yielding a reproducible amount of thrombin (identical c t) even after two months storage and (ultimately) independent of the mode of activation used.

The absence of *clot-lysis*, or even of *clot-retraction*, in the  $T_1$  series, observed for 10 days at 37 C, is noteworthy. The extremely weak (6 to 7 day) fibrinolysis in the  $T_2$  series confirms other data pointing to a trace of proteolytic enzyme in the thromboplastin preparation. The possible significance of protease contaminants received special attention throughout these studies and the tests for lytic factors will be noted in the majority of the experiments. There is no detectible trace of such factors in PRO B, nor in the bovine fibrinogen (B F).

TABLE 6—*Stability of Prothrombin in Presence of  $Ca^{++}$  and Thrombin*

$T_{Ca}$  = 15 cc PRO F (0.35%) + 5 cc buffer + 1 cc  $CaCl_2$  (M/10)

A = Activation data on thrombic mixture consisting of 2 cc  $T_{Ca}$  (1 min old at start) + 0.25 cc buffer

B = Maximal activation data, selected from series of tests (but always reached within 1 hr) on thrombic mixtures consisting of 2 cc  $T_{Ca}$  (age cited) + 0.25 tpln (var fresh preparations)

C t (in seconds), at room temp ( $23 \pm 2$  C) 0.5 cc fibrinogen (0.5% B F) + 0.25 cc thrombic mixture

Age T <sub>Ca</sub>	1 hr	1 dy	2 dy	3 dy	8 dy	Clot-lysis (37°C)
	Time in seconds					
A	250	85	43	25	4	0 (7 day)
B	4	4	4	4	4	3-4 day

*Stability of prothrombin during thrombin formation* As we shall note more fully in a subsequent section, Seegers' purified prothrombins invariably show a trace of *active* thrombin immediately on making the solution. A great deal more thrombin forms "spontaneously" on standing in solution at room temperature or in the ice-box for several days. The activating factors responsible for this will be brought out in the sequel. In the data (table 4) just considered, it is apparent that this thrombin can coexist in solution with unaltered prothrombin without demonstrable effect on the final thrombin yield. The persistence of unaltered prothrombin, still able to be activated and thus complete the (100 per cent) thrombin yield, after 22 days at room temperature, is recorded in the  $T_1^*$  footnote to table 8 A. In the following tables (5 and 6) are data to show that the same is true in the presence of amounts of (a) thromboplastin or (b) calcium salt, that are ordinarily optimal for their respective roles in the activation process.

*A Effects of thromboplastin* The addition of thromboplastin, alone, is without significant influence on the "spontaneous" activation of purified prothrombin (see table 7, T<sub>2</sub>). In the tests of table 5, 0.2 per cent PRO D is mixed with an equal volume of dilute thromboplastin (tpln A, 0.25 per cent) and kept at room temperature for three days. Equal samples were recalcified (a) immediately (20 sec)

TABLE 7—*Experimental study of certain activators of prothrombin*A *Activation series*

T 5 cc vol, containing 4 cc PRO F (0.2%) and, when indicated, ACTIVATORS, e.g. 0.25 cc of M/10 Ca or tpln A (0.25%), and 0.5 cc of tryp (40-unit) or strep (1%)—see *Reagents*  
C t (in seconds), at room temp ( $24 \pm 2^\circ\text{C}$ ) 0.5 cc BF (0.5%) + 0.25 cc T (at incubation periods stated)

T	Activators	Incubation Period room temperature											Clot lysis (3, C)
		5 m	10 m	20 m	30 m	1 hr	2 hr	4 hr	1 dy	2 dy	3 dy	4 dy	
1	(buff only)	820	726	679	681	648	517	409	246	215	181	145	0 (10 day)
2	tpln only	880	863	803	807	830	811	809	411	288	226	186	0 (10 day)
3	Ca only	192	172	134	100	65	45	26	14	13	10	7	4 day
4	Ca + tpln	92	5	4	3½	3	3	3	3	3	3	3	4 day
5	Tryp + Ca	10	6	4	4	4	6	13	27	58	140	—	3 day
6	Tryp + tpln	20	11	7½	5	5½	8	15	35	99	377	—	3 day
7	Tryp only	20	11	7½	5	5½	8	15	33	93	343	—	3 day
8	Strep + Ca	232	167	152	142	135	110	100	37	20	17	11	7 day
9	Strep + tpln	295	152	81	52	35	25	22	18	21	20	19	0 (10 day)
10	Strep only	397	325	352	360	343	330	360	197	155	90	65	0 (10 day)

B *Thrombin dilution series*

0.5 cc BF (0.5%) + 0.25 cc T dilutions (T<sub>4</sub>, 5 hr old = 1:1)

Rel T conc	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Clotting-time, in seconds	3	4½	5½	13	24	43	80	180	287

C *Second phase control Effects of Streptokinase* Same conc as in A Various thrombins (I-IV)

Thrombin	O	I	II	III	IV
	seconds				
Controls	∞	3	45	360	748
Strep	∞	3	26	230	320

and (b) after aging 24, 48, and 72 hours, respectively. At the end of the series, a control (c) was made up to contain the same amounts of prothrombin (now 72 hours older), calcium, and a fresh thromboplastin suspension. The prothrombin activation was followed in the usual way on each of the thrombic mixtures. Except for the minor difference of a slower activation in T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub> (probably explained by the thromboplastin in the mixture showing the same sort of deterioration as in ordinary solutions), the thrombin-forming ability is unchanged and results in a

final potency of 3" (c t) in every case. It can be concluded, therefore, that the prothrombin preserves its essential integrity in the presence of the crude brain thromboplastin.

TABLE 8 —Effects of Citrate and of Trypsin-inhibitors on Activation of Prothrombin

A Activation (and inhibition) data

T 5 cc vol, containing 2 cc PRO G (0.4%), with cited Activators (0.25 cc M/10 CaCl<sub>2</sub> and dil tpln D) and Inhibitors (0.5 cc vol and final strengths noted in mg per cc T)—see Reagents

Clotting times, at room temp (23 ± 2 C) 0.25T × 0.5BF (0.5%), after stated incubation periods

T	Activators	Inhibitor	Incubation Period room temperature												Clot-lysis (37 C)
			½ hr	½ hr	1 hr	2 hr	4 hr	1 dy	2 dy	4 dy	1 wk	2 wk	3 wk		
2	(buff only)	0	46m	44m	36m	33m	25m	7m	5m	4m	130"	39"	15"	0 (10 day)	
	" "	ctr (4.0)	76m	75m	76m	73m	72m	68m	69m	71m	61m	210"	75"	0 (10 day)	
3	Ca only	0	233"	210"	178"	150"	88"	23"	20"	10"	6"	4"	4"	3 day	
4	" "	T I (0.2)	220"	215"	188"	174"	115"	23"	22"	14"	8½"	5"	4"	4 day	
5	" "	S.B I (0.2)	356"	335"	340"	335"	365"	223"	280"	345"	290"	75"	21"	0 (10 day)	
6	Ca + tpin	0	5"	4"	4"	4"	4"	4"	4"	4"	4"	4"	—	3 day	
7	" "	T I (0.5)	11"	7"	5½"	4½"	4"	4"	4"	4"	4"	4"	5"	4 day	
8	" "	S B I (0.2)	120"	103"	103"	103"	118"	110"	117"	100"	43"	25"	13"	0 (10 day)	

\* After 1 hr incub with Ca + tpln, on 22nd day, T<sub>1</sub> was brought to 4" and T<sub>2</sub> to 44" (but the T<sub>2</sub> tube was contaminated by mold)

## B Thrombin dilution series

0.5 cc BF (0.5%) + 0.25 cc T dilutions (T<sub>1</sub> 3 days old 100%)

Rel T conc (%)	100	50	25	10	5	1	0.5
Clotting time, in seconds	4	8	11	22	40	84	127

## C Second Phase controls

Effects of citrate and trypsin-inhibitors (same strengths as in A) Various thrombins (I-IV)

Thrombin		I	II	III	IV
		(seconds)	(seconds)	(seconds)	
Controls	∞	5½	7	20	105
Citrate	∞	5½	7	21	110
T I	∞	5	6	19	—
S.B.I	∞	5½	7	21	—

The trace of protease in tpln A (shown by clot-lysis tests of T<sub>2</sub> in table 4) is evidently insufficient for any detectable prothrombinolysis.<sup>2</sup>

B Effects of calcium The addition of optimal amount of calcium salt causes a significant but very minor increase in the slow rate of "spontaneous" thrombin formation and in the purest prothrombins studied full activation may not be obtained even in a week. Table 6 shows such a recalcified prothrombin solution (PRO E), followed over an activation period of eight days. At any intervening period, as the data show, it is merely necessary to add adequate amount of thrombo-



plastin (three different preparations were used on successive days) in order, within less than an hour, to bring the *c t* value to the same stable optimum (4 seconds) in every case. This identical thrombin value is reached on the eighth day in the presence of added Ca, alone. Such reproducibility of thrombin *c t* value is taken to indicate (1) complete (100 per cent) activation of all the prothrombin, and (2) perfect stability of the unaltered prothrombin and of the thrombin which it yields.

*C. Conclusion as to effects of thrombin*<sup>19</sup> From these three experiments, therefore, it is significantly concluded that the presence of thrombin during the intervening period, *i e*, prior to complete activation, shows no demonstrable effects on the unaltered prothrombin, either (a) destructive (*cf*<sup>50</sup>), or (b) as an "autocatalytic"<sup>79</sup> (?) activator (*cf*<sup>1</sup>).

*Mechanism of activation of purified prothrombins* The data already considered suggest that the "spontaneous" activation of these purified prothrombins depends upon certain trace impurities. By (a) controlling individual factors discovered to participate in the activation mechanism, and (b) by testing for individual differences in behavior of the several prothrombin preparations, much detailed information can be gathered as to the factors involved.

*Experimental study of certain factors in modifying the activation of a partially purified prothrombin* Table 7 presents data on certain factors studied in relation to the activation of PRO F, which is a fairly typical representative of Seegers' prothrombin, somewhat aged.

(T<sub>1</sub>) shows very slow and incomplete (4 day) "spontaneous" activation, with buffer only, (T<sub>2</sub>) shows essentially no effect of thromboplastin, alone (the slight retardation is not very significant), (T<sub>3</sub>) shows a minor increase in activation on optimal addition of Ca-salt, alone, although the 4-day *c t* value of 7" denotes only about 20 per cent of complete activation (according to the thrombin dilution series B), (T<sub>4</sub>) shows rapid (< 1 hour) and complete activation by Ca + tpla. A (0.25 per cent).

*Pancreatic trypsin* (tryp), in a final concentration (chosen in an effort to minimize interfering proteolytic effects) of 4 "units" per cc of thrombic mixture, is an excellent activator, especially in the presence of Ca (T<sub>5</sub>). In this experiment (*cf* fig. 3), trypsin was also very effective when used alone (T<sub>6</sub>) or with thromboplastin (T<sub>7</sub>), *i e*, without added Ca. T<sub>7</sub> and T<sub>6</sub> are almost identical.

The lengthening of the clotting-times after the optimum, in these three series, is due to a *thrombolytic* action of the trypsin. Even the 1"-2" difference between the optima (as compared with the 3" *c t* optimum of T<sub>3</sub>) may be significant of some *prothrombinolysis*. Speeding up of *fibrinolysis* by a day, in T<sub>6</sub>, is significant, and the 3-day clot-lysis in T<sub>6</sub> and T<sub>7</sub> even more so (*cf* T<sub>1</sub> and T<sub>2</sub>).

*Streptokinase* (strep) was included in these experiments (T<sub>8</sub>, T<sub>9</sub>, T<sub>10</sub>) in order to control later tests (table 13) in which this agent was to be used as an activator of tryptogen (protease precursor) in certain plasma materials.<sup>33</sup>

Tests show only minor effects which can be explained in two ways, *viz* (1) some calcium content of the strep preparation, (2) some speeding up of the second-phase of the clotting process. The latter is shown in the control tests C. It is an example of the nonspecific effect of many adsorptive colloids, the action of which we refer to as *fibrinoplastic*.<sup>18</sup> In the absence of thrombin, the streptokinase has no

coagulant properties (C, O) Neither does it show proteolytic effects ( $T_9$ ,  $T_{10}$ ) but, if anything ( $T_8$ ), lessens the effectiveness of the fibrinolytic factor noted in  $T_3$

Before going on to a detailed consideration of the individual prothrombin-activators, we shall present some data on the effects of certain *inhibitors* because of the light they shed upon the activator mechanisms

*Effects of certain inhibitors on activation of purified prothrombin*

(Tables 8 and 9) *Citrate* (citr) The 'spontaneous' activation of these prothrombin solutions is very greatly inhibited by 4 mg per cc (final concentration) of trisodium citrate. Only after a week is there evidence of a trace of activation, but this is still barely noteworthy at the end of two weeks, and only 1 per cent (approximate) in three weeks (table 8,  $T_2$ ) The persistence of unaltered prothrombin, on the twenty-second day, is shown by incubating the residual  $T_1$ ,  $T_2^*$  with Ca +

TABLE 9 — *Inhibitory Effects of Heparin During Prothrombin Activation*

$T_1$  = 4 cc PRO E (0.35%) + 0.5 cc buffer soln + 0.25 cc tpln A (0.25%) + 0.25 cc M/10  $\text{CaCl}_2$

$T_2$  = 4 cc PRO E (0.35%) + 0.5 cc hep (100 unit) + 0.25 tpln A (0.25%) + 0.25 cc M/10  $\text{CaCl}_2$

Clotting tests 1a — 0.25  $T_1$  + 0.25 buffer soln + 0.5 BF (1%)

1b — 0.25  $T_1$  + 0.25 hep (10 unit) + 0.5 BF (1%)

2 — 0.25  $T_2$  + 0.25 buffer + 0.5 BF (1%)

$T_3$  = 4 cc PRO + 0.25 buffer + 0.25 Ca + 0.5 *trypsin* (10 unit), incl for future reference (table 18)\*

Thrombic Mixture (T)	5 m	15 m	30 m	1 hr	2 hr	8 hr	1 dy	2 dy	3 dy	7 dy
1a (without heparin)	63"	42"	20"	9"	6"	4"	4"	4"	4"	4"
1b (2nd phase control)	840"	60"	29"	12"	8"	5"	4½"	4½"	4½"	4½"
2. (with heparin)	2 hr	375"	60"	19"	13½"	10"	9"	8"	7½"	4½"
3 (with trypsin)*	32"	—	8"	5"	4"	4"	8"	14"	40"	—

tpln for 1 hour  $T_1$  is brought to the 'complete' c t of 4 seconds  $T_2$  fell only to 44 seconds and proved unstable (48 seconds in 4 hours), but the obvious growth of a *mold* in the solution could explain the lysis of prothrombin (and thrombin). In a very similar but unspoiled test,  $T_1$  of table 16, the citrated PRO G was brought to the full c t 4 seconds value, on the seventeenth day (v p 1149). The citrated prothrombin, from the start, shows a faint trace of coagulant activity, which is the best evidence that the prothrombin actually contains this as a trace impurity and not as the result of activation proceeding only in solution.

*Crystalline pancreatic trypsin inhibitor*<sup>22 52</sup> ( $T_1$ ) table 8,  $T_4$ , shows that 0.2 mg per cc (final concentration) of crystalline pancreatic trypsin-inhibitor is almost without effect on the activation of prothrombin by Ca-alone, while 0.5 mg per cc has only a slight delaying action on the activation by Ca + tpln.

(The enzyme-inhibitor does delay clot-lysis by about a day (37 C).)

*Crystalline soybean trypsin-inhibitor*<sup>14</sup> (SBI) in final concentration of 0.2 mg per cc, is markedly inhibitory to the activation of prothrombin both by Ca-alone (T<sub>6</sub>) and by Ca + tpls (T<sub>8</sub>). Its inhibition of the fibrinolytic enzyme is complete.

The differences between SBI and TI may be significant, suggesting that the soybean inhibitor has certain direct "antithromboplastic" (? anticephalin) effects not seen (to any important extent) with TI and probably unrelated to its effects on any protease present.

The *second phase effects* of citrate and the trypsin-inhibitors are negligible in the control tests (table 8, C).

*Heparin*<sup>31</sup> (Hep) It is important to note Seegers' claim<sup>61</sup> that his purified prothrombins are 'antithrombin'-free, and there is no reason, therefore, to suspect that heparin, as used in the tests of table 9, is acting other than directly, i.e., in the absence of 'co-factor' or 'heparin-complement'. In the amount used (100 units, = 1 mg per cc), heparin has some "antithrombic" inhibitory (clot-de-

TABLE 10—Effects of Varying Amounts of Ca<sup>++</sup> During Prothrombin Activation

T thrombin mixtures, 5 cc vol., containing (in borate buffer) 1 cc PRO H (0.1%) + 0.25 cc tpls B (0.5%) + 0.25 cc CaCl<sub>2</sub> (final strengths cited). Clotting-times (sec), at 29°C, pH = 7.7, for 0.25 cc T + 0.5 cc BF (0.5%) + 0.25 cc diluent (containing same amount of tpls B as in T and exactly enough CaCl<sub>2</sub> to bring to same final conc (0.0125 M) in each clotting (T + BF) mixture.

T	Ca in T	1 m	5 m	10 m	15 m	30 m	1 hr	2 hr (ft)
		seconds						
1	0	127	126	125	125	129	139	145
2	0.002M	104	12	4½	4	4	4	4
3	0.005M	99	6	4½	4	4	4	4
4	0.025M	98	6	5	4½	4	4	4
5	0.05 M	139	75	52	21	7½	5	4

laying) effect on the *second-phase*, which can be seen by comparing series 1b (in which heparin was added to the fibrinogen in amounts exactly corresponding to those entering the clotting mixtures in series 2 with the heparin-free mixtures of series 1a. It is noteworthy that this antithrombic action is most marked in the case of the weak initial thrombins but almost negligible (½ second) in the powerful fully-formed thrombins at the end of the series. Series 1b, therefore, is the proper control for the *first-phase* action of heparin, which series 2 shows to consist in a marked delaying ("antiprothrombic," in a general sense)<sup>18</sup> action on the prothrombin activation. After a week, however, the 4½ seconds c t is identical with the control, proving that there is no difference in the ultimate thrombin yield. These results closely resemble those obtained<sup>13</sup> by a slight reduction of the thromboplastic factor (table 11) and are best termed 'antithromboplastic'<sup>17</sup> (cf<sup>6</sup>).

(From data published elsewhere,<sup>33</sup> it is established that heparin, acting alone, is unable to inhibit the fibrinolytic enzyme.)

*Consideration of individual prothrombin-activators*

## I CALCIUM

The ability (a) of citrate (or oxalate, etc.) to prevent the 'spontaneous' activation of prothrombin solutions and (b) of added *ionized* Ca-salt to speed up thrombin formation, to a degree depending upon certain "thromboplastic" factors (see below) confirms the long-established fact<sup>11</sup> that ionized calcium ( $\text{Ca}^{++}$ ) is ordinarily essential for the activation of prothrombin to thrombin

*Amount of calcium present* Paucity of materials precluded any attempt at quantitative Ca analysis of these prothrombins, particularly since the inability to improve the extremely slow "spontaneous" activation by simply adding thromboplastin (e g table 7, T<sub>2</sub>) was believed to indicate that very little calcium could be present. However, a qualitative test, in which 1 cc of 0.4 per cent PRO G was treated with an equal volume of M/1  $\text{K}_2\text{C}_2\text{O}_4$ , showed a definite turbidity and overnight sedimentation of a trace of calcium oxalate. In this particular preparation,

TABLE 11—Effects of Varying Thromboplastin Concentration on Activation of Prothrombin, with Special Reference to Thrombin Yield

5 cc vol of (T) thrombic mixtures (A-E) containing 2 cc PRO A + 0.25 M/10  $\text{CaCl}_2$  + repln A (final dilutions stated), in borate buffer (pH = 7.7) C t (sec), at room temp ( $25 \pm 2^\circ \text{C}$ ) 0.5 B F (1%) + 0.25 T

Thrombic Mixtures	A	B	C	D	E
Conc of thromboplastin	1 500	1 1000	1 4000	1 40,000	1 400,000
Rel conc	800	400	100	10	1
Optimal thrombin c t	4	4	6	8	10
Time needed to reach optimal activation	1 hr	2 hrs	18 hrs	3 days	4 days
CLOT-LYSIS (at 37 C)	3 day	5 day	>7 day*	0 (7 day)	0 (7 day)

\* Good clot-retraction, but lysis incomplete

therefore, contamination with some available calcium was actually demonstrated. PRO G was used in the experiments of table 7, but neither in this case nor in the data of similar studies (see tables) can the test with buffer alone be taken as evidence of differences in the amount of calcium operating (as compared with the recalcified mixtures), since the thromboplastic factor is also a variable. Only when thromboplastin, alone, is added, as in T<sub>2</sub> of table 7, without improving the activation, can it be suggested that inadequate amount of available calcium (in PRO F, in this case) is responsible for the extremely slow and inadequate thrombin formation. It is not possible in the 4 day observation period of this particular experiment to predict the ultimate thrombin yields in T<sub>1</sub> and T<sub>2</sub>. The mode of action of 'thromboplastic enzyme' is discussed later (pp 1146, 1150) but it may be stated from the data of table 7 that *trypsin* appears able to make both calcium and thromboplastic factor (prob cephalin) available' from otherwise inert combinations with the prothrombin protein (T<sub>5</sub>, T<sub>6</sub>, T<sub>7</sub>)

*Effects of varying  $\text{Ca}^{++}$  concentration in first phase of clotting* (Table 10) It has long been recognized<sup>11</sup> that the essential action of Ca-ions in the clotting process is

limited to the first phase (activation of prothrombin) and requires a  $\text{Ca}^{++}$  concentration above a certain "minimum" and not in excess of a certain "optimum" because of inhibitory effects<sup>14-17</sup> The exact working out of the effects of varying the amount of calcium during first phase tests is rendered difficult<sup>70</sup> because of a significant second phase action, at least with higher Ca concentrations, due to relatively nonspecific ion effects during the thrombin-fibrinogen interaction<sup>68</sup> The experiments summarized in table 10 represent a new attempt to clarify this problem

All reagents, including  $\text{CaCl}_2$ , were dissolved in borate buffer The (added) Ca content was varied from 0.005M ( $T_1$ - $T_5$ ) and the amount of thromboplastin was kept constant throughout A series of *diluents* was prepared containing the same amount of thromboplastin and exactly enough  $\text{CaCl}_2$  for admixture with an equal volume (0.25 cc) of the respective thrombic mixtures and 0.5 cc B F to

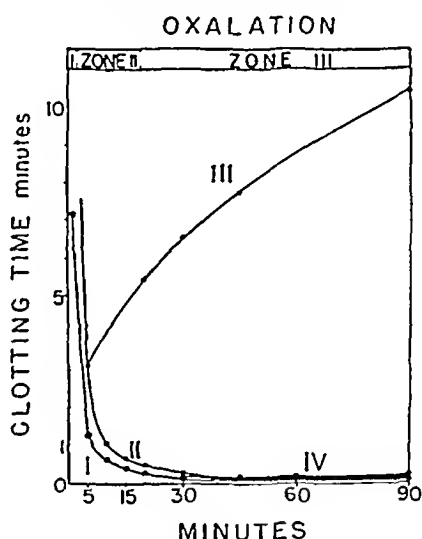


FIG. 2. EFFECTS OF OXALATE AT VARIOUS STAGES (ZONES) OF PROTHROMBIN ACTIVATION (ref

Am J Med 3 67, 1947) Clotting- and incubation-times at 25°C, pH = 7.7 (borate buffer)

I Thrombic mixture ( $T_1$ ) 13.5 cc PRO C (0.5%) 0.75 cc tpln A (0.25%) + 0.75 cc M/10  $\text{CaCl}_2$  Clotting tests 1 cc B F (1%) + 0.25 cc buff 0.25 cc  $T_1$  II Clotting tests 1 cc B F (1%) + 0.25 cc M/1  $\text{K}_2\text{C}_2\text{O}_4$  + 0.25 cc  $T_1$  III Thrombic mixture ( $T_2$ ) 3 cc  $T_1$  (5 min old) + 3 cc M/1  $\text{K}_2\text{C}_2\text{O}_4$  Clotting tests 1 cc B F (1%) + 0.5 cc  $T_2$  IV Thrombic mixture ( $T_3$ ) 3 cc  $T_1$  (30 min old) + 3 cc M/1  $\text{K}_2\text{C}_2\text{O}_4$  Clotting tests 1 cc B F (1%) + 0.5 cc  $T_3$

bring the Ca concentration in the final clotting mixture to 0.0125M in every test The control of Ca and tpln in the "second phase" is, therefore, complete The optimal 4 seconds c t was identical in  $T_2$ - $T_4$  and no difference was noted in another test, not included in the table, with  $T_3$  + buffer (instead of diluent) which reduced the Ca concentration to 0.00125M Thus, in a range of Ca concentration without second phase effects, there is seen (table 10) marked variations in the rate of prothrombin activation, but no effect on the amount (potency) of thrombin ultimately formed For this 'first phase' action of Ca salt, there is a definite optimum at about 0.005M ( $T_3$ ), with delayed activation below this ( $T_2$ ), and an inhibitory slowing at higher  $\text{Ca}^{++}$  concentrations ( $T_5$ )

*Effects of oxalate (or citrate) during prothrombin activation* The thrombin-intermediary " In 1937 we published some experiments in support of the view that

thrombin formation proceeds via an intermediary" (calcium-prothrombin-cephalin) complex or compound<sup>12</sup> Although this idea seems to have been well received (ref<sup>70</sup>), the intervening decade has not brought forth confirmatory publications from other laboratories. We have, therefore, taken the present opportunity to repeat the same kind of experiments with the newer highly purified prothrombins. Fig. 2, reproduced from a recent review,<sup>29</sup> shows what we believe to be a typical result. In this experiment, a control (1) thrombic mixture (PRO C (0.5 per cent) + Ca + tpln) was followed without oxalate. A portion of the same mixture was removed (a) after 5 minutes (3) and again (b) after 30 minutes (4), in each case being added to an equal volume of M/1  $K_2C_2O_4$  and the further activation studied, by using *double the volume* of the new mixture in the clotting-test. A second-phase control (2) consisted in adding, to the fibrinogen, a *single volume* of the original thrombic mixture shortly (60 sec.) after the addition of a like volume of M/1  $K_2C_2O_4$ . Buffer was used instead of oxalate in 1.

Under these fully-controlled conditions, therefore, it is highly significant that the earlier (5 min.) oxalation (3) of the prothrombin-thrombin mixture showed *progressive loss* of thrombic potency, in marked contrast to the "riper" (30 min.) mixture (4), which is unaffected by addition of oxalate. Our earlier paper<sup>12</sup> may be consulted for evidence that the final thrombin may be "decalcified" by oxalation and electrodialysis without significant loss of thrombic potency.

## II THROMBOPLASTIC PHOSPHOLIPID

A *Cephalin* (ceph.) The phosphatide, or more accurately the group of phospholipids (since there are isomers and homologues with varying fatty acid radicals, not all of which are clot-aiding<sup>14</sup>), designated chemically as *phosphatidyl ethanolamine*,<sup>35</sup> is a thromboplastic agent. At least this is true of the P-lipid as isolated from brain and other tissues as well as from plasma, etc., the fact being first clearly recognized by Howell<sup>36</sup> and Zak<sup>71</sup> in 1912, although the identification of the phosphatide required later work and may still be incomplete.<sup>1-4</sup> In some earlier quantitative studies<sup>14</sup> on crude (Howell-type) prothrombin, we showed (1) as little as one part of cephalin in several millions has distinct thromboplastic effects, (2) thrombin yield depends upon amount of 'free' (or 'available') cephalin present, (3) considerable amounts of cephalin (and other substances) can be demonstrated in combination with the prothrombin protein<sup>10</sup> even when it shows little, if any, ability to be activated by Ca, alone.<sup>30</sup>

It would be highly desirable to repeat these data, especially (a) the analytical and (b) the full study of thrombin yields, upon the newer prothrombin preparations. At present, however, we are handicapped by lack of cephalin and of sufficient prothrombin. With two old (several years) and weak cephalin preparations, we were able to confirm the thromboplastic action of the P-lipid, but this is the extent of the data which, for the present, we can include under this head.

B *Thromboplastin* (tpln.) We have had no opportunity to study the *thromboplastic lipoprotein* which Chargaff et al.<sup>4</sup> claim to have isolated from lung tissue and plasma. The present data were obtained with the use of various crude *thromboplastin* (*thrombokinas*) preparations described under *Reagents*. Mertz, Seegers and

Smith<sup>50</sup> found that the amount of added thromboplastin (a lung preparation) determined the thrombin yield from purified prothrombin. Table 11 shows the essential data when we follow for 7 days the effects of varying concentration of brain thromboplastin (tpln A) added to recalcified dog prothrombin (PRO A), a partially purified preparation. The least amount of the tpln in the thrombic mixture was 1/400,000 which was weak, but by no means minimal. The 1/1000 and 1/500 concentrations are clearly maximal. Owing to the limited amount of prothrombin available, there was insufficient thrombin for a dilution series, but it is clear, nevertheless, that (1) above a certain "maximum," increasing the thromboplastin concentration speeds up the *rate* of prothrombin activation without increasing the thrombin *yield* (as shown by c t value), whereas (2) below a certain "optimum," lessening the thromboplastin definitely reduces the final amount of thrombin formed, in addition to greatly slowing the rate of activation. Thus, the main facts, previously reported with regard to the thromboplastic action, whether of cephalin<sup>14</sup> or crude thromboplastin,<sup>50</sup> are adequately confirmed in the present study.

The data of table 11 again clearly show the trace of fibrinolytic factor, which, since it diminishes with the thromboplastin concentration, must come chiefly from this source.

*Deterioration of thromboplastic impurity in prothrombin preparations.* During the course of these investigations we had occasion to secure prothrombin activation data of similar kind on repeated experiments with the same prothrombin preparation over some weeks or months. It became apparent that a change in the character of the activation could often be noted. An especially good example of this is seen in data obtained, as little as three weeks apart, in the case of PRO G, as is clearly seen from the data collected in table 12. The rate and degree of "spontaneous" activation are particularly affected (1a vs 1b), but there is also much weakening of the ability to be activated by Ca, alone (2a, 2b). Even the better start which the Ca gets in a repetition (2c) of the last test, after 24 hr. keeping the PRO G solution (in ice-box), does not significantly hasten completion of thrombin formation. However, there is no appreciable difference in the response to Ca + tpln (3a, 3b), which is complete (4 seconds c t) in one-fourth to one-half hour in both instances, thus proving that the properties of the prothrombin itself are stable. The lengthening of the clot-lysis time by about one-half day in 2b, 3b hardly seems significant. The conclusion favors the idea of a deterioration of a more essential thromboplastic factor than the protease impurity. Our guess is that this refers to the thromboplastic phospholipid ('cephalin-like' in character).\*

### III THROMBOPLASTIC ENZYME

#### *Thromboplastic enzyme in relation to blood-clotting and proteolytic phenomena*

The Ferguson blood-clotting theory<sup>23</sup> centers around a special thromboplastic role of small amounts of natural plasma or tissue proteolytic enzyme (tryptase<sup>53</sup> or

\* It is possible that we were here encountering the effects of an unstable accessory factor "called *accelerator globulin*" by Ware, Seegers et al. (see Addendum).

*plasmin*<sup>6</sup> or fibrinolytic protease<sup>12</sup>) However, this is not regarded as a basic factor, since prothrombin, in our experimental systems, is rapidly and completely converted into thrombin by calcium ( $\text{Ca}^{++}$ ) and cephalin (thromboplastic phospholipid) even when no protease can be demonstrated We had shown this previously<sup>30</sup> when using crude prothrombin Trypsin, *in vitro*, corrects the thromboplastic defect of hemophilia<sup>16</sup> In the present study, all the thromboplastic preparations (except cephalin) showed at least a trace of tryptase impurity, revealed by the clot-lysis test The role of the thromboplastic enzyme we believe to be of the nature of a weak digestive action ("disaggregation," Pope<sup>57</sup>) upon the complex unions of plasma proteins with phospholipid and calcium, thereby *mobilizing* or making "available" these necessary activators and thus, in a sense, "catalyzing" the prothrombin activation The high fidelity with which the new purified prothrombins permit us to follow the course of the activation process has enabled us to contribute the following highly significant data on (1) the thromboplastic activity of natural tryptase in a variety of plasma products and (2) certain interrelationships between thromboplastic and proteolytic properties Highlights of these data are (3) use of streptokinase (*infra*), instead of the less reliable Delezenne and Pozerski<sup>66</sup> (1903) "chloroform-method," for activation of protease precursor (tryptogen) and (4) study of effects of certain recently isolated crystalline trypsin-inhibitors, of protein or polypeptide nature, from pancreas<sup>52</sup> (T I) and soybean<sup>44</sup> (S B I)—see *Reagents*

*Thromboplastic action of tryptase (streptokinase-activated tryptogen) in human, and other, fibrinogen-containing plasma fractions* Our first clear demonstration of the "thromboplastic action" of natural plasma tryptase was made with streptokinase-activated human plasma Fraction-I (H F) and the more refined fibrinogen (H Fb) obtained from the Harvard laboratories (see *Reagents*) The data of table 13 are reproduced, with minor modifications (including clot-lysis data), from *Proc Soc Exper Biol & Med* 64 312, 1947<sup>34</sup>

The preliminary *fibrinogenolytic tests* (A) show that streptokinase (strep) activates the plasma tryptogen, in H F, to a proteolytic potency comparable to that of 20-unit (final concentration = 2 units per cc) trypsin (tryp)—(see *Reagents*)

The thrombic mixtures for the activation tests (B) consist of 5 cc vol, containing 2 cc PRO B (protease-free-*vs* T<sub>1</sub>), 2.5 cc of lysate (or buffer) + 0.25 cc buffer + 0.25 cc Ca Thus, the trypsin concentration (final) in T<sub>4</sub> is only 1 unit (= 0.01 mg) per cc Obviously, the amount of protease in these activation tests is very small indeed

The activation data, in the presence of optimal calcium (added in all tests), clearly show the "thromboplastic" improvement in thrombin formation by the *lysates* A (tryptase) and B (trypsin) in T<sub>3</sub>, T<sub>4</sub>, respectively Heat-defibrinated H F (C) is used to control the possibility of some thromboplastic factor, other than the proteolytic agent, being the cause of the T<sub>3</sub> result The series T<sub>2</sub> is practically identical with series T<sub>1</sub> (Ca only) Thus, we conclude, the enzyme acts in conjunction with the trace of thromboplastin mobilized from the prothrombin preparation

We have previously noted (table 7) that the *streptokinase* (protease activator) is devoid of significant thromboplastic, thrombic, or proteolytic effects



*Other data* on streptokinase-activated human fibrinogen (H Fb), dog  $(\text{NH}_4)_2\text{SO}_4$ -pptd fibrinogen (D fb)—see *Reagents*—are essentially similar

*Thromboplastic (and thrombic) actions of III-3 and effects of trypsin-inhibitors* (see *Reagents*) Table 14, modified (by inclusion of (1) thrombin 'percentages' and (2) clot-lysis tests) from data in the above-cited publication,<sup>31</sup> shows a definite 'thromboplastic' action of the protease contained in the Harvard human plasma fraction III-3<sup>32</sup>

The c t data of the thrombin dilution series, in section B, enable us to express the degree of activation in relative thrombin 'percentages' (v p 1134) Those for the 1 hr incubation are included in section A Note

1 The 100 per cent activation by Ca + tpln ( $T_1$ ) was actually obtained in  $\frac{1}{4}$  hr and remained stable for a week or more

2 Ca, alone ( $T_2$ ), gave only 1 per cent, in 1 hr

3 Ca + enzyme ( $T_3$ ), increased this to 25 per cent, a considerable "thromboplastic" effect

4 III-3 is not an ideal tryptase preparation, because it has some "thrombic action" of its own, but the control ( $T_6$ ) shows this to be only 1 per cent, quite unable, therefore, to account for the above effect

5 Pancreatic trypsin-inhibitor ( $T_4$ ) reduces the thrombin formation to 10 per cent

6 Soybean trypsin-inhibitor ( $T_5$ ) diminishes it to less than 1 per cent, completing inhibiting the thromboplastic enzyme The question of an additional direct "antithromboplastic" action of S B I was raised on p 1142 (and see table 8) and is fully answered below

*Nomenclature* Our nomenclature<sup>27a</sup> of the fibrinolytic and thromboplastic plasma protease as tryptase (cf<sup>69</sup>) is tentative and based on the practical consideration of many similarities (despite differences in origin, etc.) to pancreatic trypsin There is no current agreement on nomenclature,<sup>32</sup> however and there are certainly several plasma proteases, e g, cathepsins,<sup>43</sup> and possibly papain like enzyme The coincidence of thrombic and proteolytic actions in III-3 might suggest a papainase (cf<sup>43</sup>), were it not for the fact that our other tryptase preparations lack the coagulant effect

*Modes of action of trypsin-inhibitors*

1 *Antitryptic effect* These known pancreatic-trypsin inhibitors were shown to inhibit plasma tryptase in fibrinogenolytic and fibrinolytic tests<sup>33</sup>

2 *Antithromboplastic effect* The later tests of  $T_5$  series (table 14) suggest that S B I antagonizes not only the protease but also the natural thromboplastic (phospholipid) factor which continues to operate in the control ( $T_1$ ) There is no good evidence for this in the case of  $T_1$  The control tests (without added enzyme) in table 8, were made with the same prothrombin (PRO G) as the table 14 series They clearly show inhibition of prothrombin activation by S B I, both ( $T_5$ ) with Ca, alone, and, more significantly ( $T_8$ ), with Ca + tpln The  $T_1$  shows negligible effects of this sort

It is concluded, therefore, that the soybean- (not the pancreatic-) trypsin-inhibitor has additional direct antithromboplastic (? anticephalin) effects, somewhat like the first-phase action of heparin (table 9)

3 *Second-phase controls* The negligible effects of trypsin-inhibitors in the second-phase are proved in the data of table 8, C An additional possibility that S B I (under certain circumstances) might serve as a co-factor for the antithrombic effects of heparin was also ruled out by some tests noted in table 15 Observe the slight immediate antithrombic effect of 25 Toronto units per cc (final concentration) of heparin on the thrombin (0.5 per cent h g) tested (in which there is no reason to suspect the presence of any co-factor) The S B I had no antithrombic action (a) immediately or (b) after 1 hr, whether tested alone or with heparin

*Other active tryptase preparations* None of the other plasma fractions studied, except the less purified *prothrombins* G and A (see table 1) and some commercial *thrombins* (e g table 20, TL<sub>6</sub>) showed coagulant activity as well as protease effects. Protease could not be detected (table 18) in the highly purified prothrombins (B—E), nor in certain thrombin preparations (table 20, TL<sub>4</sub>). It is certain, therefore, that it must be regarded as an impurity. Excepting cephalin (purified P-lipid), all the *thromboplastic agents* (see *Reagents*) tested were found to contain a trace of tryptase (cf <sup>40</sup>). In view of this ubiquity of tryptase in all but the most highly purified plasma and tissue products, it is not surprising that we were able to demonstrate both the lytic and thromboplastic (weak) effects of this protease in crystalline *serum albumin* preparations (Harvard Laboratory<sup>9a</sup>—see *Reagents*) of both human (H S A) and bovine (B S A) origin.

TABLE 12. *Prothrombin Activation Tests Effects of Aging (3 weeks)*

T 10 cc vol, containing 4 cc PRO G (0.4%) and cited *activators* 0.5 cc M/10 CaCl<sub>2</sub> 0.5 cc tpln B (0.75%), in borate buffer, pH = 7.7 PRO in 2c same as in 2b but 24 hours older (kept in ice chest)

C t (sec), at room temp (21°–22°C), for 0.5 cc B F (0.5%) + 0.25 cc T

T	Date	Activator	Incubation Period room temperature											Clot-lysis (37°C)
			½ hr	½ hr	1 hr	2 hr	4 hr	24 hr	2 dy	4 dy	7 dy	11 dy	21 dy	
1a	1-14-47	(buff only)	610"	555"	360"	210"	145"	58"	36"	26"	12"	5½"	5½"	0 (7 day)
1b	2- 6-47	" "	2760"	2660"	2190"	2000"	1500"	428"	350"	245"	130"	39"	15"	0 (10 day)
2a	1-14-47	Ca only	167"	133"	87"	45"	31"	13"	9"	5½"	4"	4"	—	2 day
2b	2- 6-47	" "	233"	210"	178"	150"	88"	23"	20"	10"	6"	4"	4"	2½ day
2c	2- 7-47	" "	126"	119"	105"	92"	60"	38"	32"	18"	6"	4"	4"	2½ day
3a	1-14-47	Ca + tpln	4"	4"	4"	4"	4"	4"	4"	4"	4"	—	—	2 day
3b	2- 6-47	" "	5"	4"	4"	4"	4"	4"	4"	4"	4"	4"	4"	2½ day
4*	1-16-47	Ca + trypt D	4"	6"	8"	11"	—	—	—	—	—	—	—	2 hr

\* Tryptase expt, uncontrolled, incub at 39°C (incl for future ref, see table 18)

*Dog plasma tryptase* Our current preparations of high-potency plasma tryptase are not yet perfected (see *Reagents*), but the preliminary data are confirmatory of those on other tryptase preparations, with a few important additions.

Table 16 shows some prothrombin (PRO G) activation data, using dog plasma tryptase (trypt D), with and without trypsin-inhibitor and other activators.

In T<sub>2</sub>, 1500 tryptase slightly improves the weak clotting in citrate (cf T<sub>1</sub>), but the most significant point is the virtual absence of activation in 1 to 2 days, in both series. The 95 per cent (approximately) unactivated prothrombin, at the end of a 17-day period, was completely converted, to 4 seconds c t, after 1 hr incubation with Ca + tpln D (see Footnote,\* table 16, A) (Fibrinolysis was advanced but not quite complete in T<sub>2</sub> series on the 7th day (37°C), whereas the control (T<sub>1</sub>) series were negative).

The thromboplastic action of the tryptase is seen in T<sub>4</sub> and T<sub>5</sub>, as compared with the T<sub>3</sub> control (Ca, only). When thromboplastin *plus* Ca are the activators (T<sub>9</sub>), the tryptase is hardly necessary, but it does reduce the total activation time from 2 to 1 hr (T<sub>10</sub>).

The fibrinolytic potency of the added trypsin is marked in  $T_3$  and evident in  $T_4$  (by completion of lysis in 1 day less than in  $T_3$ ). The same is true of  $T_{10}$  versus  $T_7$ .

Two reasons are suggested for the failure of the thromboplastic action to be as strikingly evident as the fibrinolytic effect (1) Deterioration of thromboplastic (? P-lipid) impurity, as previously noted (table 12), (2) Prothrombinolysis, in case of stronger protease  $T_6$ , as shown by the longer early c t values, together with some thrombinolysis, definitely shown in the late c t values. The failure to reach

TABLE 13 — *Thromboplastic Action of Plasma Trypsin, Compared with Standard Trypsin* I  
A. Preparation of activators. Timing of fibrinogenolysis

Clotting-time test 0.5 cc mixt + 0.25 cc thrombin, enzyme-free (h g 1%), at 24 C, after stated incubation periods, at 39 C

	(cc) Mixture	$\frac{1}{4}$ min	$\frac{1}{2}$	$\frac{3}{4}$	1	1	1½ hrs	(incub. at 39 C for lysis)
A	4.0 H F (1%)	6"	15"	45"	85"	+	$\infty$	(c t, at 24 C)
B	0.4 strep (1%)							
	4.0 H F							
C	0.4 tryp (20-u)	6"	21"	51"	90"	+	$\infty$	( )
	4.0 H F							
	0.4 buffer							

heat-defibrinated at 56C (3 min), filtered through glass wool

#### B. Prothrombin activation tests

$T_5$  cc vol, containing 2 cc PRO B (0.2%), recalcified (0.25 cc M/10  $\text{CaCl}_2$ ), and mixed with 2.5 cc of cited activators (A, B, C)

C t (sec), at 24 C, pH = 7.7 (borate buffer), for 0.5 cc B F (0.5%) + 0.25 cc T, after stated incubation periods, at room temperature

Incubation periods, at room temperature												
T	Activator	Incubation Period room temperature										Clot lysis (37 C)
		$\frac{1}{4}$ min	$\frac{1}{2}$ hr	1 hr	2 hr	6 hr	18 hr	24 hr	4 dy	7 dy		
		seconds										
1	Ca only	268	228	195	163	110	72	55	26	13	0 (7 day)	
2	mixt A	307	247	207	172	98	68	55	28	12	0 (7 day)	
3	B	324	132	86	62	45	36	28	20	12	18 hr	
4	C	335	58	40	27	13	8 $\frac{1}{2}$	7	5	5	18 hr	

less than a 13 second c t is especially significant. With the weaker trypsin, however, the first of these two reasons must be invoked. The similarity to the trypsin data of figure 3, in the section to follow, is noteworthy.

*Mode of action of thromboplastic enzyme.* We have already (p 1146) mentioned our theory that trypsin (experimentally) or tryptase (naturally) are only accessory to the basic mechanisms of prothrombin activation by calcium ions and thromboplastic phospholipid (cephalin). Tryptase, therefore, can be expected to work only in the presence of adequate amounts of the true activators. In the original presentation of the thromboplastic enzyme theory,<sup>23</sup> it was commented that the final answer must await the preparation of prothrombin entirely free from all traces of its ac-

tivators This difficult goal is, obviously, not fully achieved by Seegers' methods of purification Nevertheless, some highly significant facts emerge from a critical consideration of certain differences in behavior of individual prothrombin preparations, particularly when the preparations act in the presence of thromboplastic enzyme

*Limitations of thromboplastic action of trypsin* The differences between the data of figure 3 and table 17 are especially interesting

A Data for figure 3 were obtained on an old prothrombin preparation (PRO C) Thrombin 'percentages' (100 per cent = complete activation) were obtained from a thrombin dilution series, plotted as  $1000 - ct$  (sec) in the dotted line, and used to construct the 'activation curves' I-IV The 5 cc volume thrombic mixtures all contained 4 cc PRO C (0.3 per cent) + 0.25 cc M/10  $\text{CaCl}_2$ , with the following additions (I) 0.75 cc buffer only, (II) 0.25 cc buffer + 0.5 cc trypsin (40-unit/cc), (III) 0.5 cc buffer + 0.25 cc tpln A (0.25 per cent), (IV) 0.25 cc tpln + 0.5 cc trypsin Note the results (I) Ca (alone) produces slow activation complete in 50 hours, (II) Ca + trypt is not significantly better, (III) Ca + tpln is adequate but rather slow in the later stages, thus extending the complete activation to about 24 hrs, (IV) Ca + tpln + trypt is significantly best and completes over 90 per cent of the activation in less than 1 hour, although, here again, the penultimate stages are still rather slow and require up to 16 or 18 hours for 100 per cent completion

*Clot-lysis* was followed for 4 days, only, in which time it was absent in I, incomplete in III, and complete (24-48 hours) in II and IV Other tests failed to show any protease in PRO C (table 18)

B Table 17 also gives 'percentage' data, computed from a thrombin dilution series It gives a very good idea of the progress of the activation, under various activator conditions Omitted from the table are (1) *Control tests*, with Ca + tpln, which show the fibrinogen to be prothrombin- (and thrombin-) free, (2) Tests with PRO D and (a) buffer only and (b) + tpln only, which show only negligible traces of thrombin already present in the prothrombin solution (Weak as they were, these traces of clot resisted fibrinolysis for a week at 37 C)

Note the following results, in sharp contrast to those of PRO C (fig 3) ( $T_1$ ) a stronger tpln + Ca gave complete (100 per cent) activation in  $\frac{1}{2}$  hr, while ( $T_2$ ), a weaker tpln + Ca needs 48 hrs This is reduced by 4-u of trypsin to only 3 hrs, but ( $T_5$ ) trypt + Ca (without added tpln) is nearly as good, except in the earlier stages, ( $T_4$ ) Ca, alone, is slower to start than Ca + tpln ( $T_2$ ) but is complete in the same length of time (48 hrs) ( $T_6$ ) Trypsin, alone, produces maximal activation in 6 hours, but this amounts to only 20 per cent of the potential activity, and even this rapidly falls off in the next 24 hrs Loss of thrombic potency after maximal activation is also seen in  $T_3$  and  $T_6$  Clearly, the trypsin has prothrombinolytic and thrombinolytic powers

*Clot-lysis* fails to reveal any trypase in the prothrombin ( $T_4$ ) but there is evidently a trace in tpln C ( $T_7$ ) The tryptic fibrinolysis is readily detected ( $T_3$ ,  $T_5$ ,  $T_6$ )

The *activation tests* (with "tryptase") in tables 13 and 14 resemble those of table 17, while the data in table 16 are suggestive of those in figure 3. The significant differences in enzyme (protease) effects incidentally noted in a large, but not always complete, set of studies on PRO 'S A, B, C, D, E, F, and G, are collected in table 18. Without entering into a detailed analysis of the data it can be concluded, with considerable probability, that the differences consist in the varying amount of (a) thromboplastic phospholipid factor, and (b) calcium (especially note  $T_1$  table 7 *versus*  $T_2$  table 16) available" (1) immediately or (2) because of the actions of the proteolytic enzyme, for the prothrombin-activation. Lack of tryptase is a simple explanation of the unduly long incubation period necessary for  $Ca + tpin$  to activate highly purified prothrombins, if it can be assumed that the ac-

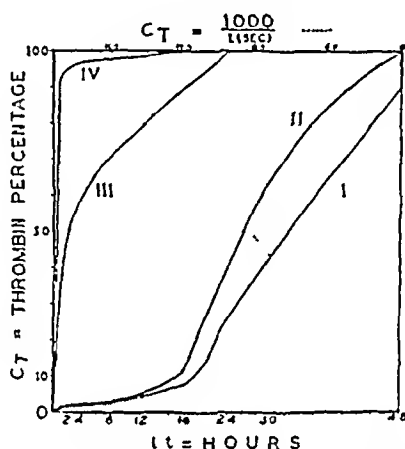


FIG 3 EFFECTS OF TRYPSIN ON THROMBIN FORMATION, FROM PURIFIED PROTHROMBIN apparently CONTAINING INSUFFICIENT TRACE OF THROMBOPLASTIN (ref ANN N Y Acad Sci 49 486, 1948)

Experimental data given in text. Thrombin formation at successive incubation times ( $t$ ) computed from inverse clotting times ( $1000 - t$  (sec)) of thrombin (III) dilution series (dotted line) for the following mixtures I PRO C + Ca, II PRO C + Ca + tryp, III PRO C + Ca + tpin A, IV PRO C + Ca + tpin A + tryp 25 C, pH 7.7

*celerator globulin* (see Addendum) is adequate. The data are all very clear in terms of the thermoboplastic enzyme theory.

*Suggested scheme for routine testing for "trace impurities" in prothrombin preparations*  
The foregoing experimental analysis of the activation mechanisms suggests four simple routine tests which should be made on all prothrombin preparations.

A 0.5 per cent prothrombin solution in our borate buffer (see *Reagents*) is recommended, with the addition of 0.4 per cent (final concentration) of trisodium citrate (except in IV). Thrombic mixtures (T) 5 cc vol, containing 2.5 cc PRO (0.5 per cent) + activators, as follows (v *infra*). Clotting-tests, at room temperature, on 0.5 cc prothrombin- and enzyme-free fibrinogen (e.g. 0.5 or 0.25 per cent B F, the "half-strength" being recommended for protease tests, I).

I. *Test for protease* (tryptase), by clot-lysis at 37 C.  $T_1$  is activated by 0.25 cc M/10  $CaCl_2$  for suitable periods (e.g., 1, 4, 24, 48 hrs), timing the clotting (at room temperature) and then incubating (37 C) for fibrinolysis. If a suitable *enzyme free* thromboplastin (e.g., 1:1000 cephalin) is available, it would greatly shorten the necessary incubation. Owing to tryptase contaminants commercial thrombo-

plastin preparations are usually unsuitable. The protease may be assayed in "fibrinolytic" units by our "method A" (p 1156)<sup>21</sup> using standard trypsin dilutions (1 mgm — 100 units) and an enzyme-free thrombin (e g 1 per cent h g — see *Reagents*) to clot the fibrinogen.

By this test, significantly, all Seegers' highly purified prothrombins, except PRO F, (table 18) are quite free from tryptase. This is definitely not the case, however, with (a) partially purified prothrombins (G, A), nor (b) with most of the currently available *thrombin* preparations (table 20).

TABLE 14 — *Thromboplastic Action of Plasma Trypsin II Human Plasma Fraction III-3 (Harvard)*  
*Effects of Trypsin-Inhibitors*

A *Prothrombin activation data*

C t (sec), at 24 C, pH = 7.7 (borate buff) 0.5 cc BF (0.5%) + 0.25 cc T

T 5 cc vol, containing 2 cc PRO G (0.4%), 0.25 cc M/10 CaCl<sub>2</sub>, 2 cc III-3 (0.1%), 0.25 cc tpln B (0.75%), 0.75 cc trypsin-inhibitor (from pancreas T I, 0.2%, from soybean, S B I, 0.2%), where indicated

T	Activators	Inhibitor	1 m	1 hr	1 hr	1 hr	%, 1 hr	1 dy	7 dy	Clot lysis (37C)
			seconds					(seconds)		
1	Ca + tpls	o	78	4	4	4	100	4	4	2 day
2	Ca only	o	218	167	133	87	1	13	4	2 day
3	Ca + III-3	o	77	20	14	10	25	8	5	7 hr
4	+	TI	71	61	39	25	10	9	6	4 day
5	+	SBI	75	92	100	108	<1	150	45	0 (7 dy)
6	Ca + III-3	o	78	—	—	80	1	87	—	17 hr

B *Thrombin dilution (percentage) data*

C t (sec), at 24 C 0.5 cc BF (0.5%) + 0.25 cc T<sub>1</sub> (3 day old), dilutions stated

Rel T conc %	100	50	25	10	5	1	0.5
Clotting-time, in seconds	4	8	11	22	41	84	127

II *Test for thrombin* Provided that the prothrombin is free from protease (or, possibly, by ensuring this by the addition (with controls) of a little trypsin-inhibitor), the addition to 0.5 cc of the test fibrinogen of 0.25 cc of *citrated* PRO is at least a qualitative test for the presence of even very minute traces of thrombin.

III *Test for thromboplastic factor* (? cephalin, ?? lipoprotein) Test I gives some indication of the immediately available thromboplastic factor, but supplementing T<sub>1</sub> with 0.5 cc of 20-unit/cc *trypsin* (see *Reagents*) before the clot-timing (T<sub>2</sub>, 0.25 cc + B F, 0.5 cc, at room temperature) test series will greatly speed up the activation (to minutes or an hour or two, instead of days) if 'available' thromboplastic factor is present. On the other hand, deficiency of this factor will be shown by the lack of difference between III and I (Ca-alone), control.

IV *Test for available calcium* This must be made in the absence of 'decalcifying'

anticoagulant "Spontaneous" activation and especially the acceleration of this by added thromboplastin (alone) is indicative of "available" calcium. The most rapid and satisfactory test, however, is to use (T<sub>1</sub>) a thrombic mixture containing PRO

TABLE 15 — *Lack of Antithrombic (second-phase) Effects of Crystalline Soybean Trypsin Inhibitor, with and without Heparin*

Ct (sec) for 0.5 BF (0.5%) + 0.5 thrombic mixtures (I-IV), consisting of 0.25 cc hg (0.5%) + 0.125 cc, each, of inhibitors noted. Tests made immediately (15 sec) and repeated after thrombic mixtures had stood at room temp (22 C) for 1 hr.

Mixture	I	II	III	IV
Inhibitor	none	SBI (0.5%)	hep (100 unit)	SBI + hep
15 sec incub	15"	15"	37"	37"
1 hr incub	15"	15"	35"	35"

TABLE 16 — *Effects of High Potency Plasma Trypsin on Aged Prothrombin Activation and Inhibition (by Crystalline Pancreatic Trypsin inhibitor)*

Activation data: Expts started 2-11-47 (cf tables 8 and 12)

T: 5 cc vol, containing 2 cc PRO G (0.4%) citrated (0.4%), and Activators (0.25 cc) and Inhibitors (0.5 cc) aged Trypsin (tryp D) used in (final) dilutions noted. Final conc (mg/cc T) of citrate = 160 of trypsin inhibitor TI = 0.5. Thromboplastin (tpln B), 0.75%, weakened after 4 days in ice box.

Ct, min (m) or sec ("), at room temp (22 ± 2 C), for 0.25 cc T + 0.5 cc BF (0.5%)

C <sub>0</sub> , mm (in) / sec ( ), at room temp (22 ± 2 °C), 10.0 ± 2.0 cc 1 + 0.5 cc D <sub>2</sub> O (5%)															
T	Activators	T <sub>I</sub>	Incubation Period room temperature												Clot lysis (37 °C)
			1/2 m	1/2 hr	1/2 hr	1 hr	2 hr	4 hr	1 dy	2 dy	4 dy	8 dy	11 dy	17 dy	
1	0	0	76m	76m	77m	78m	79m	75m	51m	49m	20m	7m	115"	39"	0 (10 day)
2	tryp <sup>t</sup> (1 500)	0	48m	47m	46m	43m	45m	44m	43m	41m	18m	5m	90"	35"	7 day
3	Ca only	0	204"	180"	149"	125"	96"	48"	25"	20"	13"	8"	4"	4"	2 1/2 day
4	Ca + tryp <sup>t</sup> (1 500)	0	183"	136"	117"	83"	54"	39"	22"	19"	9"	5"	4"	4"	1 1/2 day
5	Ca + tryp <sup>t</sup> (1 20)	0	198"	403"	360"	345"	238"	180"	19"	13"	16"	48"	59"	74"	50 min
6	Ca + tryp <sup>t</sup> (1 20)	0.5	184"	156"	135"	111"	74"	54"	27"	26"	19"	11"	7"	5"	3 day
7	Ca + tryp <sup>t</sup> (1 500)	0.5	180"	142"	145"	121"	101"	86"	36"	30"	25"	13"	7"	4"	4 day
8	Ca only	0.5	188"	177"	159"	142"	126"	112"	43"	35"	28"	20"	7"	4"	4 day
9	Ca + t <sub>pln</sub>	0	100"	14"	7"	5"	4"	4"	4"	4"	4"	4"	4"	4"	2 1/2 day
10	Ca + t <sub>pln</sub> + tryp <sup>t</sup> (1 500)	0	88"	13"	6"	4"	4"	4"	4"	4"	4"	4"	4"	5"	1 1/2 day

\* After 1 hr incub with Ca + tpln D, on 17th day, T<sub>1</sub> and T<sub>2</sub> were both brought to 4" ct value

(see T<sub>1</sub>), without calcium, but *with* 0.25 cc thromboplastin (Ca-free) and 0.5 cc 20-unit/cc trypsin (Ca-free)

By the use of these (or similar) tests, we secured the tentative data on "trace impurities" of the various prothrombin preparations noted in table 1

#### V TEST FOR ACCELERATOR GLOBULIN (see Addendum)

#### *Proteolytic phenomena in relation to coagulation processes*

I *Fibrinolysis* It is clearly proven by the data of these and earlier<sup>30</sup> studies, that thrombin itself, or its prothrombin precursor, are devoid of ordinary proteo-

lytic properties Fibrinolysis or clot-resolution occurs only when the clotting system is contaminated with some protease (e g , tryptase) of plasma or tissue (e g , *crude* thromboplastin) origin Tryptase impurity of the prothrombin itself

TABLE 17—Effects of Trypsin on Activation of Prothrombin, in Presence of Various Activators  
Thrombin dilution series See table 3

Percentage values of thrombin present, computed from clotting-time data, after stated incubation periods, in tests on prothrombin-free fibrinogen

T 5 cc vol, containing 4 cc PRO D (0.2%) and stated Activators, per cc T

T	Activators			Incubation Period room temperature									Clot lysis (37 C)
	Ca (M/10)	tpln C (0.1%)	tryp (40 u)	5 m	15 m	30 m	1 hr	3 hr	6 hr	24 hr	30 hr	48 hr	
	cc	mg	"										
1	0.05	0.15	0	40	80	100	100	100	—	—	—	—	not tested
2		0.05	0	1	5	9	20	30	35	70	80	100	6-7 day
3		0.05	4	35	42	60	75	100	50	3	1	—	3 day
4		0	0	—	±	±	+	1	4	40	80	100	0 (10 day)
5		0	4	6	25	35	60	100	80	4	1	—	3 day
6	0	0	4	—	+	<1	>1	8	20	2	+	—	3 day

TABLE 18 Some Significant Differences in Enzyme (Protease) Activator Effects on Individual Prothrombins  
Composition of thrombic mixtures and times required for complete activation (I-V)

Data cited prev	Agents, per 10 cc vol of Thrombic Mixture					I	II	III	IV	V
	Prothr	Tpln	Ca(M/10)	Protease (clot lysis)*		Calcium alone	Ca + en zyme	Ca + tpln	Ca + tpln + en zyme	tpln + en zyme
	Prep (%) vol	Type (%) vol	vol	(a) added	(b) in PRO Ca					
	cc	cc	cc	"				hr	hr	hr
Tab 4 " 8	A(?)4	A(0.1)0.5	0.5	tryp (40)	±(6 day)*	48hr	1hr	2	—	—
	B(0.2)8	A(0.1)0.5	"	" (40)	0(10 day)*	4dy	1½hr	2	—	—
	" 4	—	"	" (10)	0(7 day)*	>7dy	4dy	—	—	—
Fig 3	C(0.3)8	A(0.25)0.5	"	" (40)	0(4 day)*	50hr	48hr	24	16	—
Tab 17	D(0.2)8	C(0.1)0.5	"	" (40)	0(10 day)*	48hr	3hr	48	3	—
Tab 9	E(0.35)8	A(0.25)0.5	"	" (10)	0(7 day)*	8dy	2hr	8	—	—
" 7	F(0.2)8	A(0.25)0.5	"	(40)	+(4 day)*	>4dy	½hr	1	—	½
" 12	G(0.4)4	B(0.75)0.5	"	tryptase	+(2 day)*	4dy	(½hr)	½	—	—
16	"	"	"	tryptase	+(2½day)*	11dy	11dy	2	1	—

\* Protease in prothrombin (recalcified) tested b, time required for complete clot lysis

† Uncontrolled test, at 39 C

is ruled out in nearly all the highly purified products (table 18) studied, but is shown in those that are only partially purified (esp G, A). We are not yet fully able to account for the fact that the protease impurity always shows up best in *recalcified* thrombic mixtures. McDonald and Kunitz<sup>49</sup> have shown the importance



of calcium in the formation of trypsin from crystallized trypsinogen (pancreatic) but our experiments, to date, have not definitely proved whether this kind of action is involved in the plasma tryptase system

*Clot-retraction* is evidently a preliminary stage of fibrinolysis in our in vitro tests, where it is not at all uncommon to observe a very weak protease cause retraction of the clot without complete fibrinolysis (e g, T<sub>C</sub>, table 11) In ordinary blood clot retraction, platelets and other cellular elements<sup>53</sup> may very well contribute proteolytic enzyme(s)<sup>25</sup> We have found tryptase in platelets by means of the fibrinogenolytic test It is not improbable that colloidal syneresis, of the nature of elastic retraction due to micellar rearrangement, may also be involved in clot retraction, but a partial fibrinolysis could be an essential initiating force

In a somewhat analogous manner, a colloidal phenomenon (coacervation, Mommaerts<sup>51</sup>), characterized by interlacing of the elongated (filamentous) fibrinogen molecules and micelles, may be the funda

TABLE 19 — *Prothrombinolysis by Plasma Tryptase*

PT = 10 cc PRO H (0.1%), citrated (0.4%) + 0.625 cc Tryptase-D 2 cc. samples of PT removed at age cited and activated (T) by 0.125 cc each of M/10 CaCl<sub>2</sub>, tpin D, and borate buffer C (sec), 0.5 cc BF (0.5%) + 0.25 cc T Temp 25 C, pH 7.7

T (sec), 0.5 cc B.F. (0.5%) + 0.25 cc 1 Temp 25 C, pH 7.7								
T	Age Pt	1 m	5 m	20 m	1 hr	2 hr	6 hr (11)	Clot lysis*
		(seconds)						
	min							
1	1	136	6	4	3½	3½	3½	12-15 m (37 C), 1 hr (25 C)
2	15	292	35	7	5½	5	4	, ,
3	30	435	72	22	13	10	6	, "
4	60	612	247	123	77	48	16	, "

\* Alternate tubes tested at the respective temperatures

mental explanation of their aggregation to form fibrin, but, here again, a specific causative agent is necessary, and this is the function of thrombin Thrombin is not proteolytic (v supra) but the proteolytic enzyme papain (see p 1148), which can also produce true fibrin, also has fibrinolytic powers<sup>11</sup>

II *Fibrinogenolysis* As we have previously shown, lysis of fibrinogen,<sup>20</sup> even better than fibrinolysis,<sup>21-24</sup> is an excellent quantitative method for assaying trypsin-like enzymes, with a sensitivity of the order of 1:1,000,000 The two tests, which we term 'method B' and 'method A,' respectively, may be adapted for the study of natural proteases related to the blood-clotting system, which study is being further prosecuted in our laboratories \*

It is significant that small amounts of contaminating plasma proteases do not greatly interfere with the clotting-system In part this may be due to the wide range of fibrinogen concentrations that give optimal clotting (table 2) In certain situations, however, such as the noncoagulability of cadaver<sup>64</sup> and menstrual blood,<sup>48-65</sup> a simple explanation is forthcoming on the basis of the fibrinogenolytic action of larger amounts of tryptase, *with* or *without* thrombic clotting

\* More recent experiments emphasize practical difficulties due to differences in the shape of the lysis curves (plotted against incubation time)

III *Prothrombinolysis* is the other factor to be considered in poorly clotting blood, plasma (e g after citrated storage), or exudates. Prothrombinolysis may precede or accompany fibrinogenolysis. A simple test with thrombin will quickly show up any fibrinogen present. If this is negative, fibrinogen should be added,<sup>69, 67</sup> but otherwise this is unnecessary, before going on to a test for prothrombin by the addition of optimal amounts of calcium and thromboplastin (e g, the Quick test<sup>68</sup>).

We have long been aware of the prothrombinolytic action of trypsin,<sup>80</sup> and Seegers and Loomis<sup>60</sup> have recently noted this with tryptase ("fibrinolytic enzyme"). Some evidence of prothrombinolysis is observed with the high-potency tryptase in the activation tests ( $T_6$ ) of table 16, but the phenomenon is best seen on incubating prothrombin solution with tryptase and testing samples, at intervals, for activation with Ca + tpln (table 19). The data of table 19 clearly show the progressive weakening of the prothrombin by the added tryptase.

TABLE 20—*Thrombinolytic Action of High-Potency Plasma Tryptase*

TL thrombin-lysis mixt, containing 1 cc thrombin (type specified—see Reagents), 1 cc trypt D, and buffer (to 2.5 cc vol). Incub (of TL) and clotting tests at room temp C t (sec) 0.25 TL + 0.5 B F (0.25%). Tryptase used in 1, 3, 5, buffer only (controls) in 2, 4, 6.

TL	Thr (str)	TL-incubation period room temperature								Clot-lysis
		½ m	1 hr	2 hr	4 hr	8 hr	1 dy	2 dy	4 dy	
		seconds								
1	T <sub>U</sub> (1%)	10	10	10½	12	13	19	40	—	6-10 min (22 C)
2	"	10	10	10	10	10	10	10	11	>7 day (22 C)
3	h g (1%)	10	10½	11	12	13	17	36	—	6-10 min (22 C)
4	"	10	10	10	10	10	10	10	11	0 (7 day, 37 C)
5	T <sub>P.D</sub> (100 u)	6½	6½	6½	7	7	9	12	21	5-10 min (22 C)
6	"	6½	6½	6½	6½	6½	6½	6½	7	<7 day (37 C)

IV *Thrombinolysis*. Trypsin (pancreatic) also causes thrombinolysis akin to the natural irreversible disappearance of thrombin in serum.<sup>38</sup> We have noted, however, that this requires rather large amounts of trypsin.<sup>20</sup> Several recent workers,<sup>60, 46</sup> claim that thrombinolysis is not produced by tryptase. In the light of the trypsin data (e g, tables 4,  $T_4$ , 7,  $T_5$ ,  $T_6$ ,  $T_7$ , 9,  $T_3$ , but cf 13,  $T_4$ -with small amount of trypsin) we were rather surprised at these claims and at our similar negative tests, on a number of occasions (e g, table 14,  $T_3$ ). Very recently we have obtained some high-potency dog plasma tryptase (trypt D) (see Reagents). One of these preparations tested at 39 C (without control), in  $T_4$  of table 12, was highly suggestive, but convincing evidence of the thrombinolytic action, even at room temperature, was given by an even better tryptase preparation tested on half a dozen different thrombins, including  $T_1$ ,  $T_3$ , and  $T_9$  of table 16, and the commercial thrombins shown in table 20. The perfect stability of the controls and the potency in clot-lysis (and in fibrinogenolysis) of the tryptase are brought out in the data. One incidental finding was that thrombinolysis could not be followed beyond a certain point, because the tryptase, persisting in the lytic mixture, destroyed the

fibrinogen in the clotting-test mixture more rapidly than the erstwhile potent thrombin could clot it. A definite shortening of the fibrinolytic times in the tests prior to this, i.e., while the thrombin, though weakening, was still present, could be ascribed to the same cause. *Trypsin inhibitors* (T I and S B I) prevent the thrombinolytic effect.

V *Lysis of accelerator globulin* (see Addendum) is another possibility.

### SUMMARY

A number of high-potency purified prothrombin preparations,<sup>61</sup> in 0.2-0.5 per cent solution in borate buffer (pH = 7.7), maintain for days or weeks a stable thrombin-forming ability, whether (a) with buffer alone, (b) with brain thromboplastin suspension, (c) with  $\text{CaCl}_2$ . Nevertheless, they all contain a trace of thrombin and continue to activate spontaneously at a very slow rate. Optimal addition of Ca-salt somewhat accelerates this and usually leads to maximal (complete) thrombin formation in 2-11 days at room temperature. Except in a few cases, where ionized  $\text{Ca}^{++}$  is demonstrable, thromboplastin, alone, is without effect, but added *with* calcium, it completes the activation in a matter of minutes or hours depending principally upon the concentration used.

Experimental analysis of the activation process stresses the participation of (1) Ca-ions, (2) thromboplastic P-lipid factor (cephalin), (3) plasma and tissue trypsin (proteolytic enzyme). Each of these factors is studied in detail with reference to mode of action, optimal concentration, side-effects, and relation to inhibitors.

Inhibition of prothrombin activation may be considered under the following heads: (1) 'decalcifying' agents (e.g., oxalates, citrates, etc.), which (a) depress Ca-ionization and thus prevent thrombin formation, and (b), *under special circumstances*, reverse the process of activation, (2) 'antithromboplastic' (? anticephalin) agents, (e.g., heparin, and probably soybean trypsin-inhibitor, to some extent), (3) 'antitrypsin' agents (e.g., crystalline trypsin-inhibitors from pancreas and soybean), which inhibit the thromboplastic enzyme (accessory factor). Excess  $\text{Ca}^{++}$  slows *rate* of thrombin formation.

The evidence suggests that thrombin formation proceeds via an 'intermediary' calcium-prothrombin-cephalin (thromboplastic phosphatide) complex or compound. The amounts of (a) thromboplastic P-lipid (cephalin or 'thromboplastin') and (b)  $\text{Ca}^{++}$  determine both the rate of activation and the final thrombin yield. However, the ultimate ('ripe') thrombin owes none of its activity to the presence of any calcium or phospholipid.

The three types of activator (Ca, thromboplastin, and thromboplastic enzyme) occur as 'trace impurities' in prothrombin preparations, but Seegers' most purified materials are trypsin-free. Trypsin (and trypsin) are 'thromboplastic' only in the presence of adequate calcium and phospholipid factors, which may, however, be 'mobilized' from protein combination, including prothrombin. In this way the two basic activators are, in a sense, 'catalyzed' in their prothrombin-activating reactions.

The significance of proteolytic actions (fibrinolysis, fibrinogenolysis, prothrombinolysis, and sometimes thrombinolysis) by natural trypsin of plasma and tissue

origin is investigated and discussed in relation to the broader aspects of the blood-coagulation problem

### ADDENDUM

Since this paper was submitted for publication, an entirely new aspect of the process of prothrombin activation has been opened up by the discovery of a new clotting factor, variously designated, but which may be referred to by the term *accelerator globulin* (Ware, Seegers et al.) Dr. Seegers assures us that all the prothrombins here reported on contain an abundance of this factor (which is extremely potent). Further, the strength of our prothrombin solutions and the experimental conditions noted make it improbable that the data herewith reported will be invalidated by increasing knowledge of the new factor. One possible exception is the change in activation properties in some of the prothrombins discussed.

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The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under contract between Harvard University and the Office of Scientific Research and Development. The figures are reprinted by permission of the original publishers cited.

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# THE EFFECT OF INTRAVENOUS INJECTION OF TRYPSIN INHIBITOR ON THE COAGULATION OF BLOOD

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THE TRYPSIN inhibitor isolated from soya bean flour by Kunitz<sup>1</sup> has been shown to have anticoagulant activity in vitro <sup>2 3</sup> The present communication deals with the effects of the intravenous injection of this material into experimental animals The action on the clotting time, the prothrombin time, and the antitryptic activity of blood plasma and serum were studied

## METHODS

*Trypsin inhibitor* A crude preparation was obtained by the method described previously <sup>2</sup> Dialysis of the final preparation for twenty-four hours against saline in the ice-box was found to be necessary for the removal of a toxic factor present in the undialyzed material Without dialysis, small amounts (0.5-1 cc.) of the material regularly killed the 5-pound rabbits in a very short time (30 to 60 seconds) with cardiac standstill in diastole It is possible that the toxicity was due to the presence of potassium ions in the preparation

The final preparation was spun at 2500 rpm for 15 minutes in order to remove all insoluble material, and the pH was adjusted to 7.4 by the addition of NaOH N/10 Enough material was prepared in one batch so that all experiments except one (in which the crystalline inhibitor was used) were carried out with the same batch of material It was kept frozen at -30°C and warmed up to 37°C shortly before the injection was given

The preparation of inhibitor was assayed against crystallin trypsin (obtained from the Plaut Research Laboratory, Bloomfield, N. Y.) by the method of Anson <sup>4</sup> Twenty-five milligrams of this trypsin preparation produced 0.1137 mg. of tyrosin in 10 minutes at 25°C The addition of 0.2 cc. of the inhibitor preparation used in this work to this quantity of trypsin reduced the production of tyrosin from 0.1137 mg. to 0.0108 mg.

A small quantity of crystallin trypsin inhibitor, recrystallized 3 times, obtained by the method of Kunitz<sup>1</sup> was used in one single experiment as indicated below

*Experimental animals* 2 mongrel dogs and 3 rabbits received injections of the inhibitor preparation One additional rabbit was injected with the crystallin material The animals were anesthetized by the intravenous injection of from 25 to 35 mg. of nembutal per kg. Injections were made into the jugular vein in the dogs and the ear vein in the rabbits Blood samples were obtained by syringe and needle from the other jugular vein in the dogs and from the carotid arteries in the rabbits The blood samples were taken simultaneously with and without anticoagulant A mixture of 2 parts of potassium oxalate and 3 parts of ammonium oxalate in dry form was used as an anticoagulant Ten milligrams of the mixture was used for every 5 cc. of blood The plasma was removed immediately by centrifuging

The clotting time was studied on blood taken without anticoagulant by a modification<sup>5</sup> of the method of Lee and White <sup>6</sup>

The prothrombin time was measured on the plasma by the method of Quick <sup>7</sup>

The presence of an anticoagulant in the samples showing a prolongation of the clotting time was tested as described previously <sup>8</sup>

*In the experiments on rabbits* the samples of plasma were tested for their antitryptic activity before and after the injection of trypsin inhibitor preparation One half cubic centimeter of oxalated plasma was mixed with one half cubic centimeter, of a solution of crystallin trypsin and the proteolytic activity of

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the mixture measured on a hemoglobin substrate by incubation at 37°C for  $\frac{1}{2}$  hour, according to the method of Anson.<sup>1</sup> The crystallin trypsin was the same used for the assay of the inhibitor.

In the experiments on dogs the samples of blood serum were tested before and after the injection of trypsin inhibitor for antiproteolytic activity against the blood plasma enzyme. As shown previously, the trypsin inhibitor from soy and bean is also inhibitor towards the blood plasma enzyme.<sup>2</sup> This was done by measuring the rate of dissolution of 0.1 cc of fibrinogen solution by a chloroform plasma preparation (containing the active plasma proteolytic enzyme) in the presence of each sample of serum, as described previously.

## RESULTS

Tables 1 and 2 show the overall results obtained in the experiments on two dogs and four rabbits.

1. *Effect on clotting time* There was an immediate prolongation of the clotting time following the injection of the soy and bean preparation in dogs as well as in rabbits. This effect was transient, lasting from forty minutes to one hour, after the injection of respectively 8 cc per Kg and 5 cc per Kg into the 2 dogs, and from 30 to 60 minutes in the 3 rabbits that were followed longer than one hour.

TABLE 1—The Effect of the Intravenous Injection of Trypsin Inhibitor on Clotting Time, Prothrombin Time and Fibrinolysis

Dog #	Trypsin inhibitor (cc)	Clotting time at 37° Intervals**						Prothrombin time Intervals**						Time of lysis of fibrinogen in presence of serum from blood taken at stated intervals*					
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1 16 Kg	85	8	55	40	24	22	9	7	10	9	5	9	5	9	5	9	5	3	106
2 10 Kg	80	10	52	13	11			14	16½		16½	16½		11	180*		180*	120	76

\* 0.1 cc fibrinogen + 0.1 cc serum + 0.4 cc chloroform plasma preparation

\*\* 1 before injection, 2 from 5-10 minutes after injection, 3 from 10-30 minutes after injection, 4 from 30-40 min after injection, 5 from 46-60 min after injection, 6 from 60-90 min after injection

The injection of the small available quantity of crystallin inhibitor into rabbit #4 produced a small prolongation of the clotting time (table 2, exp 4).

2. *Effect on prothrombin time* This was prolonged following the injection into dogs and rabbits and remained prolonged for a longer period than the clotting time (tables 1 and 2).

3. *Effect on antiproteolytic activity* of blood serum and blood plasma. In the 2 experiments on dogs a chloroform plasma preparation (containing the active plasma proteolytic enzyme) was mixed with serum from blood obtained before and after the intravenous injection of trypsin inhibitor, and the mixture was tested for fibrinolytic activity on a solution of fibrinogen. Table 1 shows that the time for complete fibrinolysis of the clot increased sharply in the presence of serum from blood obtained immediately after the injection of the inhibitor. The time of fibrinolysis was still considerably prolonged at the end of the 2 experiments (table 1).

Figure 1 presents the results of experiment 1 in graph form.

In the 4 experiments on rabbits, the amount of tyrosin produced by trypsin in

TABLE 2.—*The Effect of the Intravenous Injection of Trypsin Inhibitor on Clotting Time Prothrombin Time and Antitryptic Activity of Plasma*

Rabbits #	Type of preparation	Clotting time at 37° C*, min							Prothrombin time, sec							Quantity of tyrosin produced by trypsin in pres of plasma** (mg X 10 <sup>-4</sup> )						
		1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
1 2 Kg 300	Soya bean prepar, 17 cc	10	25			13	12	11	10	15			13	13	11	1872	660			1980	1794	2238
2 2 Kg 300	Soya bean prepar, 30 cc	1	0	24					13		15					828	249	369				
3 2 Kg 300	Soya bean prepar, 20 cc	11	27		13	11	7	8	17	23		20	16	21	23	974	468		381	348	927	
4 3 Kg 100	Crystallin inhib, 45 mg in 9 cc saline	8	13		10	7	6	5	17	23		16	16	15	13	1736	300		984	1182	1182	1112

\* 1 before injection, 2 5 minutes after injection, 3 15 minutes, 4 30 min, 5 60 min, 6 2 hours, 7 3 hours

\*\* 0.5 cc oxalated plasma + 0.5 cc solution of trypsin + 5 cc hemoglobin substrate Incubation ½ hour at 37°

Solution of trypsin 30 mg in 10 cc of water in experiments 1 and 2

20 10 cc 3 4

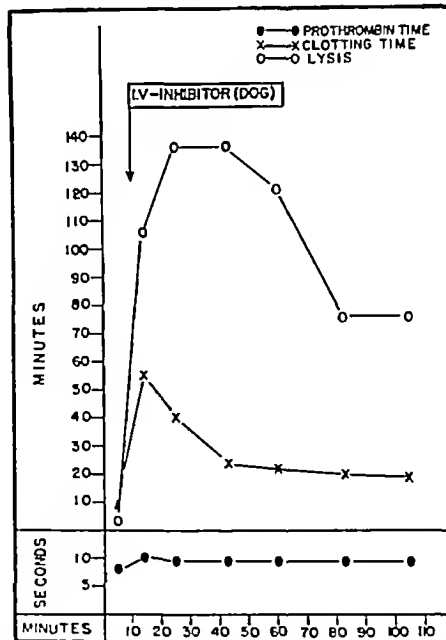


FIG 1 THE EFFECT OF INTRAVENOUS INJECTION OF SOYA BEAN PREPARATION ON ANTIPROTEOLYTIC ACTIVITY OF BLOOD SERUM, CLOTTING TIME AND PROTHROMBIN TIME

the presence of samples of plasma showed a sharp decrease with plasmas obtained after injection of the inhibitor (table 2). There was a gradual disappearance of th-



increased antitryptic effect of plasma and normal or near normal values were obtained between 1 and 2 hours after the injection Figure 2 shows the results of experiment #4 (table 2) in graph form

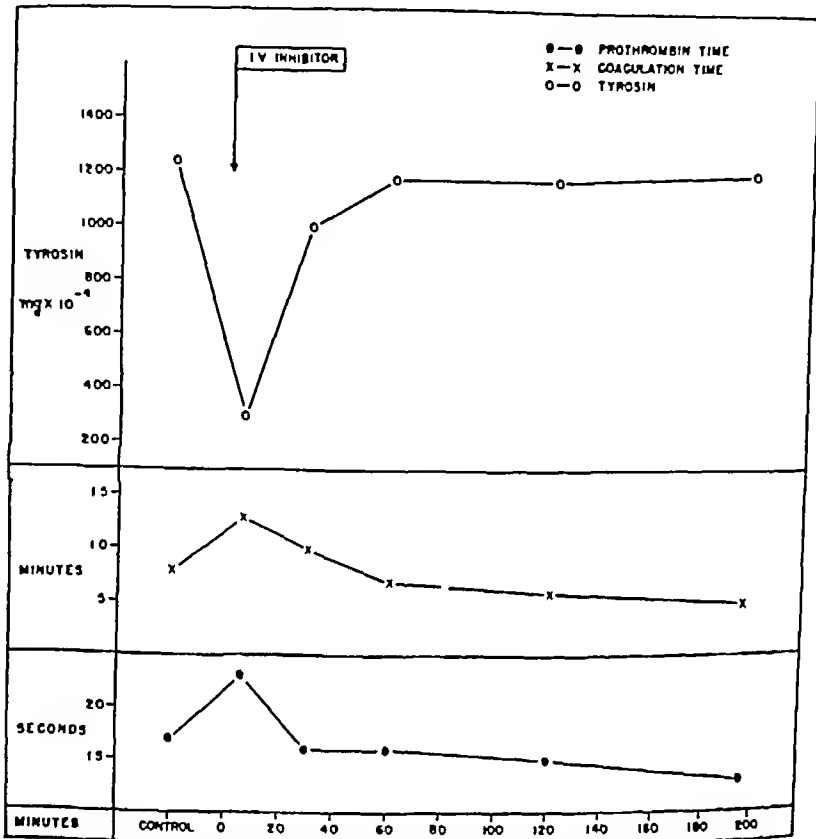


FIG 2 THE EFFECT OF INTRAVENOUS INJECTION OF CRYSTALLIN TRYPSIN INHIBITOR ON ANTI TRYPTIC ACTIVITY OF BLOOD PLASMA, CLOTTING TIME AND PROTHROMBIN TIME

TABLE 3 —Protamine Titration of Plasma after Injection of Soya Bean Preparation

One-tenth cc of citrated plasma obtained from blood showing a prolonged clotting time following the injection of soya bean preparation was added to varying quantities of protamine. The plasma was recalcified and the clotting time noted

Protamine	Clotting time at 37 C	
	Dog #2	Dog #3
0	8	10
0 01	30+	30+
0 004	30	10
0 001	11	9
0 0005	11	9
0 0001	11	7

In order to rule out the presence of heparin in blood showing a prolonged clotting time following the intravenous injection of soya bean preparation, a protamine titration was carried out on the plasma from such blood in the 2 experiments on dogs. Table 3 gives a typical example of such a titration in dog #2. The results

show that there was no significant shortening of the clotting time of the recalcified plasma by the addition of various quantities of protamin

### DISCUSSION

The data show that a soya bean preparation containing the trypsin inhibitor produced the following effects when injected intravenously into 2 dogs and 3 rabbits: it prolonged the clotting time of the blood, the prothrombin time of the blood plasma, and increased the antiproteolytic activity of the blood serum or plasma. The same effects were obtained in one experiment on a rabbit, in which a highly purified, three times recrystallized trypsin inhibitor preparation from soya bean was used.

There was some parallelism among the effects of the injection on the clotting time, the prothrombin time and the antiproteolytic activity of the plasma. This parallelism was most apparent in the experiments on rabbits because they were conducted for a longer time than those on dogs. The coincidence of the three actions appears clearly in figure 2.

The one single experiment carried out with the purified material gave results essentially similar with those obtained with the cruder material.

If one considers the fact that the trypsin inhibitor produced all these effects when added to blood *in vitro*<sup>2</sup>, it seems that the data reported here might constitute evidence that the trypsin inhibitor prolonged the clotting time *in vivo* by the same mechanism by which it prolongs it *in vitro*, and not indirectly by provoking the organism to release an anticoagulant in the blood stream. This is further confirmed by the fact that the blood showing a prolonged clotting time following the injection of the trypsin inhibitor did not contain any heparin in the two experiments in which a protamin titration was carried out. It is well known that the prolongation of the clotting time following the intravenous injection of peptone or of antigen in sensitized animals is due to the appearance of heparin in the blood of such animals.

Three different trypsin inhibitors so far have been reported as having anticlotting properties: the trypsin inhibitor from pancreas,<sup>10</sup> from blood serum,<sup>10</sup> and that from soya bean.<sup>2</sup> These three substances differ chemically and their similar action on the blood coagulation mechanism parallels their similar action on trypsin. It is to be noted that the trypsin inhibitor from soya bean and from pancreas also inhibits the proteolytic enzyme of blood plasma.<sup>11, 12</sup> The exact role of the plasma enzyme in blood coagulation is unknown: a recent communication presents evidence that the enzyme has no clotting activity by the usual tests.<sup>12</sup> It is nevertheless interesting to note that these substances which inhibit the proteolytic action of the enzyme also are anticoagulant agents.

The practical use of the trypsin inhibitor from soya bean for anticoagulant therapy must await further study. The material does not appear to be toxic and could conceivably be used, in purified form, for prolonging the clotting time *in vivo* when such action is desired. However it is quite possible that the inhibitor is antigenic and this point should be clarified before an attempt at practical application is made.

## SUMMARY

1 The intravenous injection of a soya bean preparation containing a trypsin inhibitor into 2 dogs and 3 rabbits produced the following effects: prolongation of the clotting time and of the prothrombin time, and increase in the antiproteolytic activity of the blood plasma or serum

2 Identical results were obtained in one experiment in which a crystallin trypsin inhibitor from soya bean was used

3 The significance of these results is briefly discussed

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## FIBRINOLYSIS ITS MECHANISM AND SIGNIFICANCE

By R. G. MACFARLANE, M.D., AND ROSEMARY BIGGS, M.B., Ph.D.

### INTRODUCTION

THE BIOLOGIC processes that produce a gross physical change have always attracted much interest. The transformation of fluid blood to solid clot, for instance, has been the subject of experiment and discussion the amount of which seems disproportionate to its fundamental physiologic importance. There is, however, a converse to this process of coagulation which, though equally obvious in its result, has been comparatively neglected, possibly because in its extreme form it occurs only rarely and with apparent irregularity. This is the phenomenon of fibrinolysis or dissolution of blood clot that, though normally slow, may occur with great rapidity under certain conditions. Though there has been relatively little work on this subject recent developments have made it possible to appreciate part of the mechanism that underlies it, and to realize that factors are involved which are of basic importance. It is apparent also that the process of fibrinolysis is linked with other subjects previously without obvious association, and this paper, though it cannot claim to be a complete review, is presented as an attempt to assemble relevant information, and to relate it to some observations of our own.

### DEFINITION

For the purpose of this communication fibrinolysis is taken to mean the aseptic dissolution of fibrin brought about by the direct action of a mechanism existing in normal blood. Usually dissolution may take days or weeks to complete, but may be so accelerated as a result of natural changes occurring in the living subject or of experimental procedures as to occur within a few hours or minutes. This increased activity is the main subject dealt with, and though a particular set of factors have been studied as being apparently those mainly responsible, it is recognized that others may be involved.

### THE RECOGNITION OF FIBRINOLYSIS

It is an ancient observation that circumstances may modify the permanence or stability of blood clots. Zimmermann (1846) found that ox fibrin suspended in salt solution remained intact for up to 10 days, while fibrin from human blood obtained by wet cupping dissolved in 12-24 hours. He refers to earlier observations of a similar nature by Denis (1838). Green (1887) also studied the disappearance of fibrin in saline, which occurred without obvious bacterial action, and found that once it had disintegrated fibrin could not be made to clot again by adding thrombin. Similar observations on the blood of dogs subjected to hemorrhage were made by Distre (1893, 1894 a & b, 1895), who, apparently the first to use the term fibrinolysis, came to the conclusion that fibrin is digested and not dissolved, though he did not favor the idea of a lytic enzyme in blood. Rulot (1904) believed

From the Radcliffe Infirmary, Oxford, England

that leukocytes were concerned in the digestion, since an increase in their concentration hastened the reaction. In 1905, Nolf began a series of experiments in which he induced fibrinolytic activity in dogs by various procedures, including hepatectomy and peptone shock. He regarded this work as demonstrating the participation of a proteolytic enzyme in the process of blood coagulation to which he believed fibrinolysis was a logical, if unusual, sequel. Morawitz (1906) studied the familiar phenomenon of the fluidity of the blood in cases of sudden death. He observed that blood from such cases contained itself no fibrinogen, and was capable of destroying the fibrinogen and fibrin of normal blood. Hirose (1934) supported Nolf in regarding the lysis of fibrin as the result of coagulation and also considered that it was the cause of clot retraction.

In 1937, Yudin stimulated a considerable new interest in fibrinolysis by its practical, if rather macabre, application to a blood transfusion service in Russia. It was the practice to use the blood from fresh corpses, and for obvious reasons the most suitable donors were the victims of accidental or sudden death rather than those who had died from a long drawn-out disease. This selection of subjects proved to have another advantage, for in these cases of sudden death the blood removed from the body, though it clotted in the usual way, reliquified within a few hours and could be used for transfusion without anticoagulant. Since it appeared that shock might be a relevant factor in Yudin's subjects, Macfarlane (1937) investigated the occurrence of fibrinolysis in living patients who had undergone surgical operation. There was an occasional instance of rapid lysis of whole clotted blood, but this was a rare and apparently capricious occurrence. It was found, however, that fibrin prepared from diluted plasma disappeared rapidly in a high proportion of post-operation cases, though similar preparations made before operation were stable for weeks, an observation that was soon confirmed by Imperati (1937) and later by Kaulla (1947). Mole (1943) and Wexler and Ellis (1944) confirmed the occurrence of fibrinolytic activity in the blood of cases of sudden death. Though Smith and Smith (1945) and Wilson and Munnell (1946) have reported that blood taken during menstruation shows fibrinolytic activity, which also occurs, according to the latter workers, in hypertension and eclampsia, the technic used makes it difficult to assess the significance of these observations. Tagnon, Levenson, Davidson and Taylor (1946) have observed fibrinolysis in human cases of severe burns and in one case of fatal narcotic poisoning, and have been able to induce similar activity in the blood of dogs by severe hemorrhage. They quote an observation by Ham that fibrinolysis sometimes follows the intravenous administration of typhoid vaccine in human beings.

#### THE MECHANISM OF FIBRINOLYSIS

From these scattered observations it is clear that rapid dissolution of fibrin occurs following a variety of disturbances to the living organism which may be due to an acceleration of the normal, slower process, or to some other agency. The fact that lysed fibrin is irreversibly altered, coupled with the observations of Morawitz (1906) and Mole (1943) makes it probable that fibrinolysis is the result of proteolytic digestion, and it would be logical to trace the recognition of a proteolytic

enzyme in normal blood. Since fibrin in sterile whole blood usually remains intact for some days or weeks it follows that such an enzyme, if it exists, must be under normal conditions relatively inert, though capable of activation.

#### ACTIVATION BY CHLOROFORM

In 1889 Denis and Mirbeau found that a thermo-labile proteolytic agent developed in serum after the addition of chloroform, ether or thymol, apparently the first observation of a phenomenon since much studied. Delezenne and Pozerski (1903) investigated this reaction further, showing that serum after admixture with chloroform developed the power of digesting gelatin and casein, an activity that was inhibited by the addition of untreated serum. They came to the conclusion that a proteolytic enzyme present in the serum was normally inhibited by some other substance which was removed or destroyed by chloroform, a suggestion that could be related to previous observations by Hildebrandt (1893) and Hahn (1897) that normal serum is capable of inhibiting proteolysis by trypsin.

Jobling and Peterson (1914) came to the conclusion that the antitrypsin of plasma was soluble in lipid solvents, a view which has recently been revived by Ungar (1945). Dale and Walpole (1916) and Yamakawa (1918) confirmed the destruction of the inhibitor by chloroform, and Opie, Barker and Dochez (1911) found that the administration of chloroform by stomach tube to dogs resulted in protein digestion occurring in serum samples, and apparent destruction of fibrinogen. Nolf (1921 a & b, 1922) found that proteolytic activity accompanied by fibrinolysis could be generated in mammalia and bird plasma by the action of chloroform. The action has been extensively investigated by Tagnon (1942) and Tagnon, Davidson and Taylor (1942), mainly from the point of view of its relation to blood coagulation. They found that the globulin fraction from chloroform serum was strongly fibrinolytic, and might destroy fibrinogen before clotting could occur.

#### FRACTIONATION OF PLASMA

The fact that separation of the plasma proteins may result in proteolytic activity has also long been recognized. Hedén (1904b) fractionated ox serum by ammonium sulphate precipitation, and found that the globulin contained a proteolytic enzyme that was inhibited by a thermo-labile factor associated with the albumen fraction. Opie and Barker (1907) confirmed these findings, and observed the similarity between the globulin enzyme and leuko-protease. More recently Feissly (1942) and Macfarlane and Pilling (1946a) have studied the fibrinolytic activity of globulin fractions separated by acid precipitation. Taylor et al. (1945), who have studied plasma fractions provided by Professor Cohn and Dr. Edsall, have observed spontaneous proteolytic activity associated with fractions 1 and 111-2. It is probable that fractionation separates an enzyme-inhibitor complex, analogous to the dissociable trypsin-antitrypsin compound studied by Hussey and Northrop (1923). The relationship in the latter case obeys the mass-action laws, and dissociation may occur following simple dilution of the complex, a fact which is also relevant to the plasma enzyme and its inhibitor.

Separation of the enzyme and inhibitor has also been accomplished by the use

of trichloroacetic acid Schmitz (1937) found that the enzyme was precipitated by this agent, while the inhibitor remained in the supernatant fluid. He came to the conclusion that fibrinolysis was due to the adsorption of the enzyme together with a kinase onto fibrin. Tyengar and Sehra (1942) have also utilized trichloroacetic acid in the estimation of enzymatic activity of plasma in a variety of conditions. Mole (1943), working with blood from cases of sudden death, found that the rapid fibrinolysis was due to the action of an enzyme precipitable by trichloroacetic acid. This enzyme resembled trypsin in some respects, but was shown to be significantly different in others.

Separation of the enzyme has also been accomplished by adsorption onto fibrin. Barker (1908) studied the proteolytic enzyme associated with fibrin, and Rosenmann (1922, 1936, 1937) has used lysed fibrin as the source of an enzyme preparation that he has studied extensively, finding it apparently identical with the agent present in the globulin fraction and active at pH 7.3-7.8. Macfarlane and Pilling (1947b) have also utilized the adsorption of the enzyme by fibrin. The globulin fraction of plasma is added to a solution of fibrinogen subsequently clotted by thrombin. The resulting clot is removed, washed free of protein, and allowed to lyse in saline when the enzyme is released into solution.

Following fractionation by electrophoresis most of the enzyme is found in fraction 111<sup>2-3</sup> (Cohn et al. 1944, Edsall, 1947).

#### ACTIVATION BY STREPTOKINASE

The procedures described above have produced activation of the plasma enzyme by destruction of its inhibitor, or by separation of the enzyme-inhibitor complex. Recently, observations have been made that provide an important link between fibrinolysis occurring as a result of these manipulations or of natural causes in the living subject, and fibrinolysis produced by the culture filtrate of certain strains of *β*-haemolytic streptococci. In 1933, Tillett and Garner had shown that human plasma, if allowed to clot in the presence of such filtrates, underwent very rapid fibrinolysis. The plasma of patients who had recovered from streptococcal infections and rabbit plasma resisted this lysis. They found, however, that if rabbit fibrin was clotted by human thrombin it became susceptible to lysis by the streptococcal filtrate. Milstone (1941) showed that pure fibrinogen, even if derived from human blood, yielded fibrin which was resistant to the action of streptococcal filtrate, but that if a small amount of the globulin fraction from human plasma were added lysis occurred as with whole plasma. He also found that the addition of such globulin to rabbit plasma resulted in lysis of this fibrin by the filtrate. He therefore postulated a lytic factor present in the globulin of human blood which was necessary to sensitize fibrin to the action of the bacterial enzyme. In 1944, Kaplan came to the conclusion that Milstone's 'lytic factor' and the proteolytic factor activated by chloroform were identical. This suggestion was confirmed and extended by Christensen (1945) and Christensen and MacLeod (1945). These workers showed that the enzyme liberated in plasma or serum by the action of chloroform was the same as that which appears following the addition of streptococcal filtrate. The enzyme is proteolytic, being capable of digesting fibrin, fibrino-

gen, casein and gelatin and, though it resembles trypsin, is distinct from it. It is inhibited by the so-called trypsin inhibitor of plasma and by the pancreatic anti-trypsin of Kunitz and Northrop (1936). The enzyme is present as an inert precursor associated with the globulin fraction of the plasma and its inhibitor is associated with the albumen. They postulated that streptococcal filtrate contains a kinase capable of activating the precursor so that the inhibitor is overwhelmed. Kaplan (1946) confirmed the distinction between the plasma enzyme and trypsin by showing that the streptococcal kinase did not activate trypsinogen nor did entero-kinase activate the plasma enzyme. Macfarlane and Pilling (1946a) in observations in which plasma and plasma fractions were treated with streptococcal filtrate and chloroform, came to the conclusion that plasma normally contains not only the enzyme precursor and its inhibitor, but free enzyme. They showed that simple dilution of whole plasma will, if there is an increase in free enzyme, result in the development of activity, resulting from dissociation of the enzyme-inhibitor complex.

### TERMINOLOGY

In the course of previous work on the proteolytic factor of serum during the past 40 years, different terms have been applied to what is almost certainly the same substance. This has led to confusion and it is desirable that a unified terminology should now be adopted. Such names as serum trypsin, serum protease, serum tryp-tase, fibrinolysin, thrombolysin and others should be abandoned in favor of a more specific designation. The nomenclature proposed by Christensen and MacLeod (1945) seems to fulfil the necessary requirements. In this the proteolytic enzyme of the plasma is called "plasmin," a choice for which good reasons are given. The precursor of plasmin is termed "plasminogen," and the streptococcal filtrate factor previously called "fibrinolysin" now becomes "streptokinase." The antibody developed by patients recovering from streptococcal infection, which is capable of neutralizing streptokinase, is called "antistreptokinase." The inhibitor of proteolytic enzymes present in normal plasma might be called "antiplasmin" for the sake of uniformity, though such a name implies a sense of restriction with regard to its activities which is undesirable. An alternative nomenclature proposed by Loomis, George and Ryder (1947) in which the term "fibrinolysin" is applied to the plasma enzyme, seems to us to be fraught with danger of confusion since this term has, in the past, been applied to the streptococcal factor.

### THE PROPERTIES OF PLASMIN

The identity of plasmin with the enzyme activated by chloroform apparently is established (Christensen and MacLeod 1945). The enzyme studied by Mole (1943) in cases of sudden death, and that occurring in the plasma of patients after operation or other disturbance (Macfarlane and Pilling, 1947b) are so similar to plasmin with regard to their physical properties and activity that it may be assumed, as a working hypothesis, that fibrinolysis, whether induced by streptokinase, chloroform, fractionation, or disturbances in the living subject, is due to active plasmin.

It has already been shown that plasmin is capable of lysing fibrin, and of digest-



ing fibrinogen, gelatin and casein. With regard to fibrinolysis, though preformed fibrin may be slowly digested activity is greatly increased if the enzyme is present during the process of coagulation, probably because of the strong adsorption of plasmin by fibrin during clotting. Though plasmin is capable of digesting other proteins, its fibrinolytic action is more rapid in proportion to such digestion than is the case with trypsin and other proteolytic enzymes. Fibrinolysis, therefore, is a delicate indicator of plasmin activity which has been used by Macfarlane and Pilling (1946a) for quantitative estimations. This method allows, however, of only an arbitrary expression of activity, which, in this case has been in terms of the greatest dilution of the enzyme that will produce lysis within 24 hours at 37°C of the clot formed by a 0.075 per cent solution of pure fibrinogen.

Other methods of determination of activity used by previous workers include estimation of the increase in nonprotein nitrogen following incubation of the enzyme with a particular substrate, which, in some cases has been merely whole serum, in others casein, gelatin or hemoglobin. Viscometric determinations have been made with casein and gelatin, but we ourselves have found this technic unreliable, even under rigidly controlled conditions. Estimation of acid soluble tyrosine by the method of Anson and Mirsky (1937) has given satisfactory results. We have found, by this method, that plasmin is capable of digesting all the proteins of normal plasma, a fact which may have some significance in the physiologic effects of its activity.

The pH of optimum activity of plasmin is about 7.4, and of maximum heat stability between 7 and 7.4. The enzyme is destroyed at this pH by heating to 55°C for twenty minutes. It is nondiffusible through cellophane and behaves, with regard to precipitation, like the globulin of plasma. It is precipitated by half saturation with ammonium sulphate, full saturation with sodium sulphate or by bringing the pH to 5.5 after dialysing the plasma free from electrolytes. It is precipitated by trichloroacetic acid, and by acetone and alcohol with moderate loss of activity, and it is strongly adsorbed by fibrin.

#### INHIBITION OF PLASMIN

Antiplasmin, the natural inhibitor of plasmin, is associated with the albumin fraction of the plasma. It is said not to be precipitated by trichloroacetic acid, but Duthie (1947) does not agree with this statement. The inhibitor is nondiffusible through cellophane and heat labile, being destroyed by heating to 66°C. It is rapidly inactivated at pH values below 5.0, but is stable in the alkaline ranges. It is destroyed by chloroform, ether, alcohol and acetone (Duthie 1947). As with pancreatic antitrypsin and trypsin, the plasmin-antiplasmin complex appears to be dissociable by simple dilution unless there is a considerable excess of inhibitor (Macfarlane and Pilling 1946a), and simple fractionation of the plasma into albumin and globulin is also apparently capable of splitting the complex. The activity of the inhibitor must be considered in any study of fibrinolysis, since the equilibrium it maintains with plasmin may be disturbed by changes in either factor. We have attempted to estimate antiplasmin activity by means of the inhibitory effect of the albumin fraction to be studied on the digestion of fibrinogen and casein.

by purified plasmin Plasmin is also inhibited by the crystalline antitrypsin of Kunitz and Northrop (1936) by heparin and by the soya bean trypsin inhibitor of Kunitz (1946), the latter being most effective Grob (1943) has reviewed the literature on the antiproteolytic factor of serum

#### OTHER ENZYMES POSSIBLY RELATED TO PLASMIN

The characterization of plasmin has not, at present, been sufficiently specific to determine whether or not certain other enzymes, which have been described in body fluids and tissues, are identical Since it is possible that these enzymes may enter the blood stream or, like plasmin, become activated from inert precursors, they may be briefly considered

Hedin (1904a) identified two proteases in spleen tissue, one acting at a slightly alkaline pH and inhibited by serum, as was the "leucoprotease" of Opie and Barker (1907) There has been a large number of publications by Abderhalden (1921, 1940 etc) and his associates dealing with the presence of various proteolytic enzymes in the blood and tissues which they believe to be specific for abnormal tissues Patients with carcinoma, for instance, produce enzymes specifically digesting carcinomatous tissue, and in pregnancy there is an enzyme capable of lysing placental tissue It is probable that part, at least, of these findings depend on the activation or inhibition of plasmin, but the whole subject of the Abderhalden reactions is too voluminous to be dealt with adequately here Grassmann and Heyde (1930) have described peptidases in animal serum or plasma active between pH 7 and 8 that split leucylglycyl glycine which are increased in various pathologic states Rosenmann (1936) has extracted a fibrinolytic enzyme from liver, kidney, lung, pancreas and thyroid tissue closely resembling his "thrombolysin" Maschmann (1942) has found that rats with sarcoma may have a higher level of blood peptidase than is normal Huggins, Vail and Davies (1943) have studied fibrinolysis in menstrual fluid finding considerable activity which is also present in extracts of endometrium, prostate and thyroid tissue Zamechick, Stephenson and Cope (1945) have found that lymph from the extremities of dogs contains an aminoxopeptidase which is increased in amount if the limb is traumatised or burned They have also found that blister fluid from burns in human cases contains the same enzyme, but Macfarlane (1943) failed to find fibrinolytic activity in blister fluid Beloff and Peters (1945) have investigated a protease extracted from human skin active at pH 6-8 This enzyme is not trypsin and does not split leucylglycyl glycine, and following burning its concentration in the affected skin is decreased Beloff (1946) has studied an inhibitor of this enzyme present in the albumen fraction of normal plasma, but considers that this is distinct from antitrypsin It is not clear, however, that this conclusion is justified Fruton (1946) who has made an extensive study of the tissue enzymes has found two leucine aminopeptidases in lung, serum and skin extracts

Macfarlane and Pilling (1947b) have examined human tissues for the presence of fibrinolytic activity The tissues were perfused, if practical, with running water until the vessels were blood free, minced, extracted with saline and the enzyme precipitated with ammonium sulphate or acid The precipitates were dissolved

and tested for fibrinolytic activity. This varied from one sort of tissue to another, and from case to case, but, in general, it was found that lung extract had the greatest activity being, in many cases, relatively greater than the blood plasmin activity of the same subject. This lung enzyme resembled plasmin both as regards its physical properties and its reaction with the inhibitors, but its action on pure substrates has not been investigated. Fibrinolytic activity was also observed in extracts of kidney, suprarenal and thyroid but in a lesser degree, and it was found that in all tissues the highest yields occurred in cases of sudden death when the blood plasmin level was also increased (fig. 1).

The inhibitory power of the albumin fraction of organ extracts have also been estimated (fig. 2). It has been constantly found that spleen extract has a potent inhibitory effect on plasmin and lung extract resembling that of antipiasmin. This

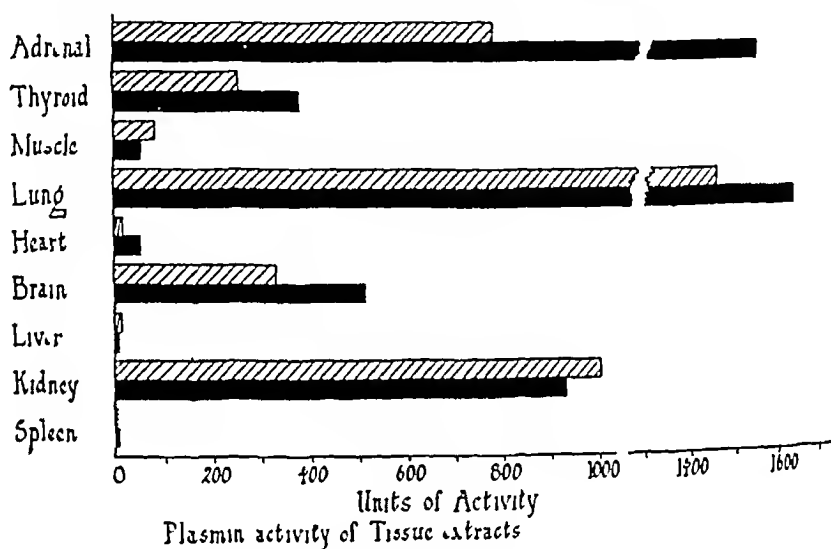


FIG. 1. Histogram showing the mean plasmin activity of extracts from various tissues and organs (a) from cases of sudden death (solid columns) (b) cases of slow death (hatched columns). The unit of activity is based on the titre of the enzyme (see text).

finding is of interest in view of the work of Ungar (1945) in which the injection of splenic extracts raised the antitryptic power of the serum.

It has been observed that there is a fibrinolytic enzyme in normal urine (Macfarlane and Pilling 1947a). This enzyme again resembles plasmin in its properties. Though the concentration of this enzyme in the urine is not closely correlated with the plasmin level of the blood, and though it differs slightly from plasmin in being heat stable and more affected by trypsin inhibitors, it is possible that it is, in fact, plasmin in the process of elimination. Other enzymes resembling pepsin have been described in urine, the literature being reviewed by Farnsworth, Speer and Alt (1946).

#### THE ACTIVATION OF PLASMIN IN VIVO

Though normal plasma may be activated *in vitro* by the procedures described previously, in its natural state it possesses no demonstrable proteolytic activity,

though this may develop following a variety of circumstances disturbing to the living subject. The train of events connecting these stimuli with the fibrinolytic mechanism is obscure, but since the nature of the latter is becoming clearer a more systematic investigation of the process has become possible, since it is probable, though not definitely established, that fibrinolytic activity *in vivo* is due to plasmin.

In his early experiments, Nolf (1905, 1908) was able to produce fibrinolysis following peptone shock in dogs in which the liver had been isolated from the circulation, thus demonstrating that this organ is not a necessary link in the chain of events leading to activation. Loeper et al (1932) found that after ligation of the renal arteries in dogs there was a loss of protein and a rise in nonprotein nitro-

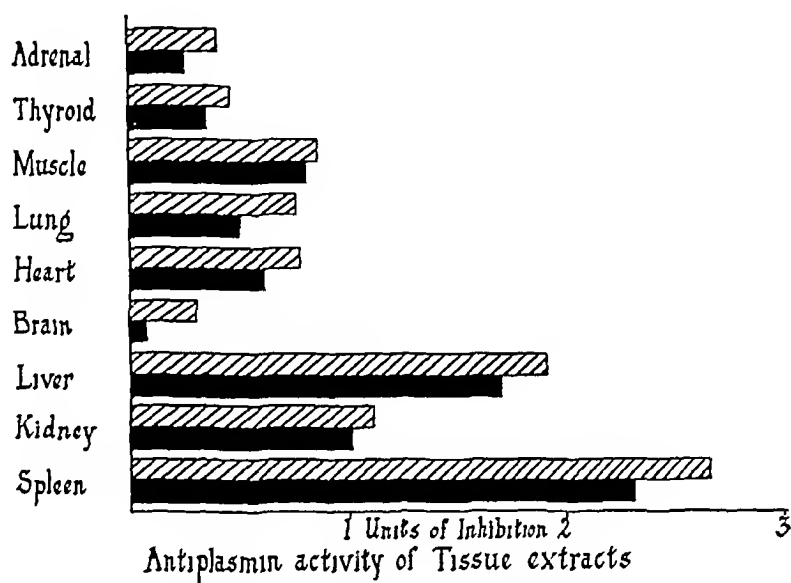


FIG. 2. Histogram showing the mean antiplasmin activity of extracts of various tissues and organs (a) from cases of sudden death (solid columns) (b) cases of slow death (hatched columns). The units refer to a dried albumin standard, and do not compare to the units in Figure 2.

gen in serum during incubation. Hougardy (1934) has made the significant observation that removal of the pancreas does not reduce the proteolytic activity of the serum when treated with chloroform.

Since it seemed that the condition of traumatic shock had existed in all cases in which fibrinolysis had been demonstrated and might be the prime mover of the process, Macfarlane (1937) investigated the blood of patients undergoing surgical operation. The technic employed used diluted citrated plasma clotted by calcium chloride, it being found that in normal blood samples the fine fibrin web so formed was stable under aseptic conditions for a period of weeks. In about 70 per cent of the patients, after operation these fibrin webs disappeared in twenty-four hours or less, though control samples taken the day before from the same patients showed no undue instability. The nature of the cases showing such fibrinolysis gave little information as to the factors responsible for this change. There was no close correla-

tion between fibrinolysis and the traumatic shock experienced by the patient since quite minor operations might be accompanied by relatively intense activity, whereas major surgical procedures might give negative results. Similar results were obtained by the same technic by Imperati (1937), though the rather lower incidence of 50 per cent of fibrinolysis following operation was recorded. He, too, observed the lack of correlation between the severity of the operation and the degree of fibrinolytic activity. From these investigations it appeared that fibrinolysis is dependent on some factor commonly associated with the procedure of surgical operation, though not obviously connected with shock or trauma, but the design of the original experiment was not sufficiently controlled to allow of its identification by analysis of its results.

A further experiment was therefore carried out (Macfarlane and Biggs 1946) in which serial blood samples were obtained from each patient to control the effect of premedication, and the anaesthetic, as well as the operation itself. It was found that a high proportion of positive results were obtained before the operation, and even before the anesthetic or premedication, which could not be explained by the pathologic condition from which the patients were suffering, since trivial conditions involving no constitutional disturbance were included. The one common factor seemed to be preoperation anxiety, a conclusion which was borne out by the independent observations of Lattner (1947) who had found fibrinolytic activity in cases of anxiety states, and hyperthyroidism in a normal person during an air raid. Kaulla (1947) found that fibrinolysis occurred after the injection of novocain or even a large volume of saline, and though he believed that displacement of tissue fluid was the activating agent in this latter experiment, the effect of anxiety cannot be excluded. In addition to the operation cases it was found that positive fibrinolysis occurred in a variety of pathologic states in which hypersensitivity appeared to be the common factor, but in view of the probable effect of anxiety, such findings are not easy to assess.

The next observations (Biggs, Macfarlane and Pilling 1947) concerned the discovery that severe exercise induced fibrinolytic activity in normal subjects. This was proportional to the amount of exercise taken, and to the degree of exhaustion it induced, the latter being influenced by training. The occurrence of fibrinolysis both in exercise and in anxiety suggested, of course, that adrenalin activity might be an important underlying factor, and experiment established that intense fibrinolytic activity could be induced in normal subjects by the injection of adrenalin. The findings resembled closely those provided by exercise, since in both fibrinolytic activity appeared in the circulating blood within a minute or so of the onset of the stimulus, and disappeared within a few minutes of its cessation, and in both there was a change in the blood picture consisting of an almost immediate rise in lymphocytes, with a slower rise in polymorphonuclear leukocytes and platelets. These results provided an explanation of previous observations, since in all cases in which fibrinolytic activity has been recorded it is likely that adrenalin activity will have been stimulated. It must be determined, of course, what part adrenalin plays in the reaction. We have made observations on this matter, but they are far from complete at present. Adrenalin itself produces no proteolytic

activity of plasma *in vitro*. It is clear, therefore, that it requires the cooperation of one or more factors which exist in the body, but not in the shed blood. The obvious possibility that the rise in leukocytes or platelets were directly responsible for the increase in fibrinolytic activity has been disproved by the finding that addition, separately or in combination, of fresh lymphocytes, polymorphonuclears or platelets to normal plasma is not followed by fibrinolysis, nor does the incubation of adrenalin with these elements result in any activating factor being produced. The plasma from subjects who have developed fibrinolytic activity retains this activity after centrifuging at 20,000 rpm to remove all its formed elements. Moreover, in patients with marked polymorphonuclear leukocytosis, lymphocytosis, or thrombocytosis, no spontaneous fibrinolytic activity has been observed.

The injection of adrenalin in certain pathologic states has given some information, if largely negative, regarding the other factors concerned. A normal fibrinolytic response has been obtained in two cases of Addison's disease in which subsequent postmortem examination has demonstrated almost complete destruction of the suprarenal cortex, and one case of aplastic anemia, in three cases of splenectomy (performed for traumatic rupture) and in three cases of hemophilia. The only patients with a constantly diminished response to adrenalin are those in whom the lymph glands have been affected by Hodgkin's disease or x-ray irradiation. From these results, and those previously mentioned, therefore, it appears that given the stimulus of adrenalin, fibrinolysis is independent of the normal function of the suprarenal cortex, spleen, pancreas, or liver, but it is possible that lymphoid tissue may be involved.

The demonstrable changes that occur in the components of the proteolytic system of plasma concurrently with spontaneous activation consist of an increase of plasmin that suggest activation of plasminogen. This rise may be sufficient in extreme cases to produce fibrinolysis in the whole blood, but usually it is necessary to dilute the plasma to demonstrate the increased activity. A more puzzling finding is the apparent diminution in the inhibitor that also occurs in spontaneous activation. Figure 3 illustrates the reduction of the inhibitory effect of the albumen fraction of normal plasma, as compared with plasma from the same subject following activation by adrenalin. It appears, therefore, that the process of activation *in vivo* is more complex than the mere secretion into the blood stream of a kinase capable of activating plasminogen. Such a kinase, however, is probably concerned, and it is of interest that Astrup and Permin (1947) claim to have observed its presence in fresh tissues, though it is not clear that they have eliminated the active enzyme already present in such preparations.

#### THE SIGNIFICANCE OF FIBRINOLYSIS

The balance between proteolysis and its inhibition may control many of the vital processes in which blood constituents are involved. The most obvious of these are the proteolysis of pathologic fibrin deposits and the destruction of plasma proteins following the activation of plasmin. In the more complicated mechanism of blood coagulation, both proteolytic and inhibitory factors can alter the speed of clotting and some naturally occurring anomalies may be related to changes in the

plasmin-antiplasmin complex. A normal balance of proteolytic and inhibitory substances in the blood may also be necessary for the dynamic equilibrium between blood and tissue proteins which probably forms the background of protein metabolism, and the negative nitrogen balance which follows alarming stimuli such as trauma, burns, etc., may be related to the plasmin activity with which they are frequently accompanied. Proteolysis may also, under certain circumstances, lead to the liberation of harmful products of protein digestion, and the release of histamine in anaphylactic shock has been attributed to this cause.

### THE DISSOLUTION OF FIBRIN

In local inflammation or trauma, fibrin is formed in the tissues, and during healing its necessary reabsorption is probably due either to the action of plasmin or

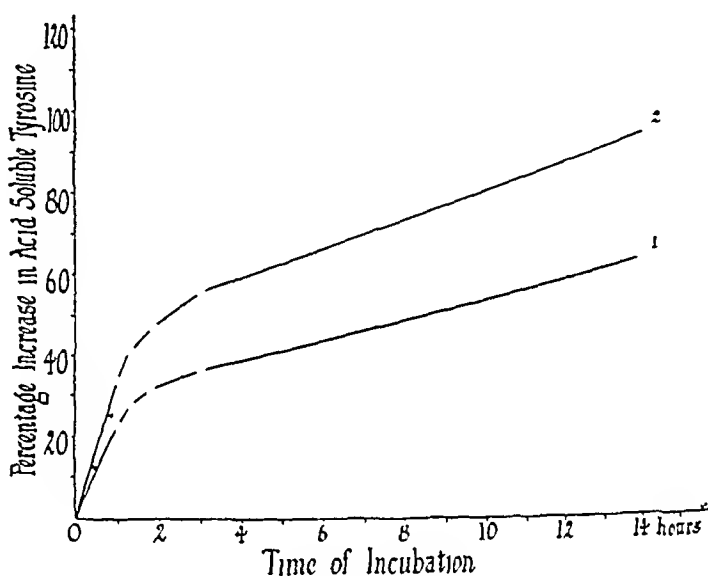


FIG. 3. Curves showing the digestion of casein by plasmin in terms of the release of acid soluble substances estimated as tyrosine. In each case a similar concentration of the albumin fraction from the blood of the same subject (1) resting, and (2) after adrenalin injection, has been added. A reduction in antiplasmin effect is observed following the administration of adrenalin.

to the proteolytic enzymes of the tissues. Similarly in pneumonia relatively large amounts of fibrin are liquified during resolution, and it is significant that the lung often contains an unusually high content of fibrinolytic enzyme. Following intravascular thrombosis the thrombus may disintegrate, a process that may be accompanied by general fibrinolytic activity which Kaulla (1947) has demonstrated in the blood of patients with thrombosis during clinical improvement. The dissolution of clots by fibrinolytic enzymes in the endometrium may be a factor in normal menstruation (Smith and Smith 1945).

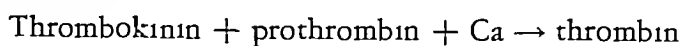
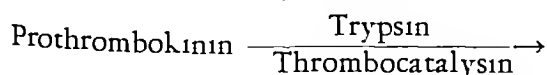
### CHLOROFORM POISONING

The destruction of fibrinogen by plasmin can seldom be demonstrated *in vivo* because the normal liver replaces fibrinogen with great rapidity so that when the blood of dogs is replaced by defibrinated blood, normal coagulation is restored within an hour (Whipple 1914, Goodpasture 1914). In chloroform poisoning,

which might produce plasmin activity from destruction of antiplasmin, there is a demonstrable fall in fibrinogen in five hours (Whipple 1914) and a profound deficiency in three days (Whipple and Hurwitz 1911, Whipple 1914, Smith, Warner and Brinkhouse 1937). This disappearance of fibrinogen, though usually attributed entirely to failure of regeneration, is more rapid than that which occurs without a toxic factor, as for instance, when the normal liver is excluded from the circulation (Whipple 1914), or following transfusion in a patient with afibrinogenemia when fibrinogen could still be demonstrated in the circulation after eight days (Pinniger and Prunty 1946). Denys and Marbaix (1889) were the first to suggest that the loss of nitrogen which occurs in chloroform poisoning might be due to proteolysis. Opie, Barker and Dochez (1911) showed that there was an increase in nonprotein nitrogen in the incubated serum from an animal with chloroform poisoning, and Jacoby (1900) demonstrated fibrinolysis in severe phosphorus poisoning. It is therefore probable that proteolysis is a factor in the loss of fibrinogen in chloroform and phosphorus poisoning.

#### BLOOD COAGULATION

There is a considerable accumulation of evidence in favor of the view that one or more components of the fibrinolytic system are concerned in blood coagulation. The fact that chloroform serum causes the clotting of fibrinogen solutions originally suggested to Nolf (1908, 1938 etc.) that proteolysis might be a factor in normal blood coagulation. He considered that two substances, thrombogen and thrombozyme combined in varying proportions with fibrinogen to form a clot, an excess of thrombogen and thrombozyme leading to the release of a proteolytic enzyme which might cause incoagulability from destruction of fibrinogen or coagulation followed by fibrinolysis. The coagulant action of the globulin fraction observed by Tagnon (1942) suggests the participation of plasmin in blood clotting, and although its exact role has not been determined the facts available suggest an analogy between the action of plasmin and trypsin. Neither purified plasmin nor trypsin will clot fibrinogen solutions (Dale and Walpole 1916, Seegers and Loomis 1947), and both apparently lead to the release of thrombin from oxalated plasma (Dale and Walpole 1916, Stephen and Wohl 1921). A further study of trypsin in relation to coagulation is therefore of interest. Ferguson and Erickson (1939) have shown that trypsin causes an increased rate of conversion of prothrombin to thrombin, and Lenggenhager (1946) showed that pure prothrombin is converted into thrombin by trypsin only in the presence of an additional plasma component which he has called prothrombokinin. The amount of thrombin formed by trypsin is directly proportional to the amount of prothrombokinin present. He suggests that trypsin activates prothrombokinin to a substance called thrombokinin which combines directly with prothrombin in the presence of calcium to form thrombin. Lenggenhager extracted a natural activator of prothrombokinin (thrombocatalysin) from plasma and found that this was fibrinolytic. According to Lenggenhager, thrombin formation proceeds as follows:





Lenggenhager's views are supported by various observations Feissly (1942) obtained a proteolytic component from the "acido-globuline" fraction of Dala dilhe (1937) which had the power to convert prothrombin to thrombin in the presence of calcium. He concluded that this substance was one of two components of thromboplastin, the second being a phospho-lipoid. Fantl and Nance (1946) and Owren (1947) both showed that an additional factor from the plasma was necessary for the conversion of prothrombin to thrombin.

The fact that plasmin and trypsin can coagulate oxalated plasma in the absence of added calcium makes it difficult to accept coagulation by these substances as closely analogous to that occurring normally. However, Ferguson and Erickson (1939) have shown that optimum coagulation with trypsin requires the addition of both calcium and cephalin, and Lenggenhager (1946) showed that trypsin even in concentrations of 0.25-1 per cent will not coagulate plasma which is continually in motion and suggested that its coagulant action on citrated plasma was due to a local accumulation of calcium ions released by proteolysis from an inactive form in combination with protein. Moreover, although chloroform serum will coagulate plasma and fibrinogen, there is some doubt as to whether purified plasmin has this power (Macfarlane and Pilling 1947b). Thus calcium may be necessary for coagulation with both trypsin and plasmin. The difficulty raised by Rosenmann (1937), who showed that substances probably identical with plasmin and anti-plasmin could be distinguished from all known coagulant factors, does not preclude the possibility that plasmin may activate an additional plasma factor not recognised by him. The present evidence, therefore, supports the view that plasmin may be concerned in the activation of prothrombin, a suggestion which implies that one of the components of thromboplastin is proteolytic.

Indirect evidence for the proteolytic theory of thromboplastin was suggested by observations on the coagulation defect in hemophilia. Tagnon, Davidson and Taylor (1942) showed that the hemophilic globulin could not be activated by chloroform, Feissly (1942) showed that the globulin fraction was not proteolytic, and Ferguson (1939) showed that hemophilic prothrombin could be converted into thrombin in the normal time on the addition of trypsin. These observations appear to suggest a deficiency in the proteolytic component of thromboplastin, but the experiments of Tagnon and Feissly could not be confirmed by Macfarlane and Pilling (1947b), and Tagnon's results have been refuted by Lewis et al (1946). Furthermore if hemophilic patients receive an injection of adrenalin they develop a normal fibrinolytic reaction with only a slight reduction in the coagulation time of whole blood and recalcified plasma (Biggs, Macfarlane and Pilling 1947).

A study of the inhibitors of proteolysis also supports the view that proteolysis is a factor in coagulation. Ferguson (1942) and Grob (1943) showed that pancreatic trypsin inhibitor delays coagulation, and Grob (1943) showed that the delay results from an inhibition of either prothrombin or thromboplastin. Macfarlane and Pilling (1946b) and Macfarlane (1947) studied soya bean trypsin inhibitor and showed that its inhibitory effect on blood coagulation was antithromboplastic. By inference these observations suggest that thromboplastin has a proteolytic component.

Inhibition by the albumin fraction in hemophilia was studied by Feissly (1944). He showed that whereas the coagulation time of whole blood in hemophilia is greatly prolonged, that of the hemophilic "globulin fraction" is only slightly longer than normal. He then showed that hemophilic albumin retards the clotting of both normal and hemophilic "globulin fractions". These experiments, which suggest an increase in the inhibitory system in hemophilia, are supported by the work of Tocantins (1943) who postulates an increase in antithromboplastin.

A direct relationship between a shortening of the coagulation time and fibrinolysis occurs following adrenalin injections. Cannon and Mendenhall (1914 a & b) showed that adrenalin stimulation of the sympathetic nervous system and emotional stimuli lead to a shortening of the coagulation time of recalcified plasma. This hastening of coagulation is apparently not due to any change in prothrombin because Wakin et al (1946) have shown that adrenalin causes no alteration in the Quick prothrombin time. It therefore seems possible that the change in coagulation time may be related to an increase in thromboplastin. Since there is now considerable evidence that thromboplastic action is in part proteolytic, and since adrenalin injections consistently produce fibrinolysis, it seems possible that this shortening of the coagulation time may be related to fibrinolysis.

#### THE ALARM REACTION OF SELYE

The suggestion that fibrinolysis occurs in all conditions which lead to secretion of adrenalin has interesting implications in relation to Selye's concept of the alarm reaction (Selye 1946 etc). According to Selye, exposure to any noxious or excessive stimulus is followed by a uniform pattern of pathologic changes. There is an initial shock phase, with the characteristics usually associated with this term, and a succeeding counter shock phase when the phenomena of shock are reversed. The reaction is accompanied by involution of the lymphoid tissue, hypertrophy of the adrenal cortex, and excessive secretion of adrenal cortical hormones.

The marked negative nitrogen balance of the alarm reaction which may be related to plasmin, has been studied in detail following trauma (Cuthbertson 1930, 1932, 1935, Vaughan et al 1947), burns (Cope et al 1943, Taylor et al 1943, Chanutin and Gjessing 1946), operations (Chanutin et al 1938), hemorrhage (Elman 1944), and infections (Coleman and DuBois 1915). There is an extensive loss of nitrogen from the tissues, an immediate and progressive fall in plasma-albumin, and from the second or third day, a rise in plasma-globulin which often continues until the albumin-globulin ratio is reversed. The early fall in plasma-albumin might be due to fibrinolysis initiated by adrenalin. The continuing destruction of protein reserves might be related to the excessive secretion of adrenal cortical hormones which are known to cause mobilization of globulins from the tissues (White and Dougherty 1945). The reversed albumin-globulin ratio thus produced might be associated with an imbalance of the plasmin-antiplasmin complex, causing further catabolism of protein.

#### *Fibrinolysis and Anaphylaxis*

Rochas e Silva et al (1946 a, b & c) have suggested that fibrinolysis may play an important part in anaphylaxis. It is well known that intravenous injections of

trypsin cause profound shock (Tagnon 1945) Rochas e Silva and Grana (1946a) showed that trypsin perfused through the isolated liver causes the release of histamine On the other hand an anaphylactic antigen caused no release of histamine from the livers of sensitized animals unless it was perfused in whole blood Since anaphylactic shock is associated both with a rise in blood histamine and fibrinolysis (e Silva and Teixeira 1946b) they suggested, that, in contact with sensitized tissue, the anaphylactic antigen releases fibrinolysin which in turn releases histamine from the liver cells In 1947 Ungar showed that incubation of sensitized tissue with the specific antigen does cause a release of proteolytic enzyme and in 1945 he showed that resistance to trauma was associated with a decreased release of histamine from the cells and an increase in blood antitrypsin These observations clearly support the original suggestion of Rochas e Silva

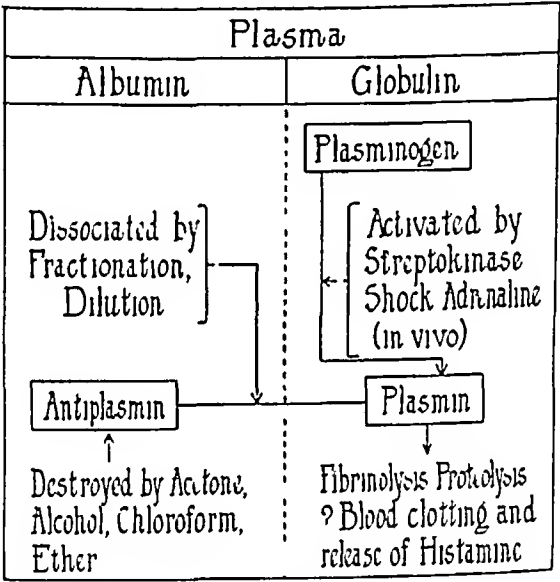


FIG 4 Diagrammatic representation of the activation of plasmin and its effects

There is, therefore, cumulative evidence that the plasmin-antiplasmin complex plays a fundamental part in several essential physiologic processes

CONCLUSIONS

- 1 There exists in normal blood a proteolytic enzyme "plasmin," its precursor ' plasminogen' and its inhibitor 'antiplasmin,' the latter being in excess
- 2 Plasmin and plasminogen are associated with the globulin fraction of the plasma, antiplasmin with the albumin
- 3 Plasmin activity can be produced in plasma ' in vitro' by the action of streptokinase which activates plasminogen, by chloroform which destroys antiplasmin, or by procedures, such as fractionation of the plasma, which separates the plasmin antiplasmin complex
- 4 Active plasmin will cause fibrinolysis, digestion of the plasma proteins and casein and gelatin

- 5 Enzymes resembling plasmin have been found in various tissues, particularly lung and in urine
- 6 Inhibitors resembling antipiasmin have also been found in various tissues, particularly the spleen
- 7 Plasmin activity may occur in the living subject following various stimuli, such as trauma, fear, severe exercise, or the injection of adrenalin
- 8 The mechanism of this activation is still obscure, and involves factors not present in whole blood. It can be obtained in the absence of the normal function of the liver, pancreas, spleen and suprarenal cortex
- 9 Such plasmin activity may be concerned in the phenomena of shock, in the equilibrium between protein breakdown and synthesis, in the mechanism of blood coagulation, and in allergic reactions
- 10 The position may be illustrated diagrammatically as shown in figure 4

#### ACKNOWLEDGMENT

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# HEMORRHAGIC DIATHESIS ASSOCIATED WITH THE PRESENCE OF AN ANTICOAGULANT IN CIRCULATING BLOOD CASE REPORT AND LABORATORY STUDIES

By J P SOULIER, M D , AND M BURSTEIN, M D

THERE ARE three observations of the presence of an anticoagulant in the blood of patients with a hemorrhagic diathesis in the American literature<sup>15, 16, 4</sup> The following case report constitutes the fourth observation of such an occurrence

The patient was studied at the "Hospital des Enfants Malades" by Dr Maurice Lamy, under whose direction this work was carried out

M B , age 21, male, was admitted to the hospital in July, 1946, with a diagnosis of hemophilia Past history revealed the frequent occurrence of hemorrhagia episodes, the first one occurring at 1½ years of age There were oral, nasal, and gastro intestinal hemorrhages, but most episodes were articular, and occurred in the absence of any significant trauma These hemorrhages required blood transfusions on two occasions

Joint hemorrhages were particularly frequent, repeating themselves 5 to 6 times in a year They affected mostly the elbows, knees, and ankle When seen, the patient was confined to bed for the last 2 months with involvement of both knees and hematuria

The family history is not contributory He was the only child and his mother died of tuberculosis The maternal grandmother, her children and grandchildren were always in good health The paternal grandfather was healthy His father had frequent episodes of epistaxis

Physical examination revealed a pronounced muscular atrophy, affecting the inferior limbs mainly, a hemophilic arthritis, and a malformation of the uvula No hematomas in the muscle The remainder of the physical examination was normal There was no syphilis

The blood coagulation time was 6 hours (at 37 C), the bleeding time 4 minutes The red cells count was 4.1 million with a hemoglobin of 75 per cent

The leukocyte count was 9,800 with the following differential count: neutrophils, 76 per cent, eosinophiles, 2 per cent, lymphocytes, 16 per cent, monocytes, 6 per cent

On Aug 2, 1946, the patient received a transfusion of 300 cc of blood (fresh) The following day, the clotting time was still 6 hours The patient was sent home where he was treated with distilbestrol He was readmitted Oct 31 In the interval period he had no severe hemorrhage episodes There were a few ecchymoses and gingivorhagiae The clotting time was 4½ hours and the bleeding time 17 minutes The tourniquet test was negative and the capillary resistance measured by the cuff method was found normal (no petechiae after 6 minutes under a pressure of 90 mm of mercury) Examination of the blood gives the following results: Red Cells, 5.1 millions, Hemoglobin, 15 g 3, Hematocrit, 50, MCV, 97.3 µ, White Cells, 6,400, Platelets, 300,000 of which 90 per cent were round forms, Fibrinogen, 400 mg per 100 cc, Calcium, 9.9 mg per 100 µ

The purpose of this communication is to report the different studies carried out on the blood of this patient The blood was obtained from the antecubital vein with and without anticoagulant The anticoagulant used was a 4 per cent solution of sodium citrate, 1 cc of which was used for every 9 cc of blood Plasma was obtained by centrifuging

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This case is the subject of a report under the title "Sur un anticoagulant présent dans le sang d'un sujet se présentant cliniquement comme un hémophile" by M Lamy, M Burstein and J P Soulier, to be published in the Rev d Hématologie

## 1 Anticoagulant action of noncitratd plasma on normal whole blood

Blood was obtained from the patient without anticoagulant. The red cells were allowed to sediment for 3 hours, and the supernatant plasma separated. Increasing quantities of this plasma were added to 1 cc of normal human blood. Table 1 shows that the patient's blood plasma had a marked ant clotting effect on normal blood.

TABLE 1—Clotting Time of 1 cc of Normal Blood to Which the Patient's Plasma Was Added (Experiment Done on Nov. 14, 1946) To 57 C Test Tubes 13 x 110 Duplicate Readings

Patient's plasma cc	Normal blood #1 (1 cc) Clotting time (minutes)		Normal Blood #2 (1 cc) Clotting time (minutes)	
0	8	10	10	15
0.1	26	33	45	48
0.2	120	180		

TABLE 2

Patient's citrated plasma (cc.)	Normal citrated plasma (cc.)	Clotting time (minutes)
0	0.2	4
0.1	2.9	6½
0.1	1.9	17
0.1	0.9	35
0.1	0.4	60
0.1	0	240+

TABLE 3—Howell's Time of Patient's Citrated Platelet-free and Platelet with Plasma (0.2 cc plasma plus 0.2 cc  $\text{CaCl}_2$ )

Date	Number of platelets (per mm <sup>3</sup> )	Clotting time (minutes 1)	Type of plasma
11-7-46	115,600	32	Platelet rich
	225	360+	Platelet free
11-14-46	125,000	45	Platelet rich
	340	360+	Platelet free

## 2 Anticoagulant action of citrated plasma from the patient's blood on citrated plasma from normal blood

Citrated normal plasma was obtained by spinning citrated normal blood at 6,000 rpm for 30 minutes. Increasing quantities of this plasma were added to a fixed quantity of citrated platelet-free plasma from the patient's blood. Recalcification of the mixture was done by adding an equal volume of 0.025N  $\text{CaCl}_2$  and the clotting time (Howell's time) measured. Table 2 shows a typical experiment performed on Sept. 14, 1946. The results can be explained only by the presence of an anticoagulant in the patient's plasma.



### 3 Clotting time on recalcification (Howell's time)

#### a Effect of platelets on Howell's time

Platelet-rich citrated plasma was obtained by spinning citrated blood at 2000 rpm for 3 minutes. Platelet-free plasma was prepared by spinning the platelet-rich plasma at 6000 rpm for 30 minutes, and passing the supernate through an Iena #4 filter. The filtrate was platelet-free for all practical purposes. Table 3 shows the results obtained by recalcifying both plasmas. The Howell's time was considerably prolonged when the platelets were removed. Similar results were obtained by Quick with hemophilic plasma. It is noticeable that the Howell's time of this patient's plasma, devoid of platelets, approximated very closely the clotting time of whole blood. However, the clotting time of platelet-rich recalcified citrated plasma was much shorter, which may indicate the existence of a destruction of the platelets during recalcification and liberation of thromboplastin from the platelets.

#### b Effect of destruction of platelets on Howell's time

It is well known that the clotting time of citrated recalcified plasma (Howell's

TABLE 4—*Howell's Time before and after Destruction of Platelets*

Date	Clotting time of recalcified citrated platelet rich plasma from patient's blood (Minutes)		
	Intact platelets	Platelets destroyed by freezing	Platelets destroyed by adding distilled water
7-25-46	90	10	15
8- 1-46	90	12	15
8- 3-46	100	20	
11- 7-46	32	20	22
11-14-46	45	13	14

time) is shortened if the platelets are destroyed and allowed to release their thromboplastin—according to Tzanek and Burstein<sup>2</sup> citrated plasma clots faster if, before recalcification, it is frozen and then thawed. They found that this procedure shortened the clotting time of citrated recalcified plasma from 3–6 minutes to  $1\frac{1}{2}$ – $2\frac{1}{2}$  minutes. One can also destroy the platelets by the addition of 3 volumes of distilled water to one volume of citrated plasma. Iso-osmoticity is then re-established by adding one volume of a 3.6 per cent solution of sodium chloride. We have studied the effect of the destruction of platelets on the Howell's time of citrated platelet-rich plasma from the patient's blood.

Table 4 shows that there is a considerable shortening of the clotting time after destruction of the platelets. However, this clotting time is still abnormally prolonged.

Control experiments on platelet free plasma showed no effect of freezing on the clotting time. However, the addition of distilled water to the platelet free plasma resulted in a shortening of the clotting time. This effect may be due to dilution of the anticoagulant by the distilled water since the addition of saline instead of distilled water produced a similar effect.

#### c Effect of dilution on Howell's time

The effect of dilution was studied platelet free citrated plasma from the patient's blood was diluted with saline and then recalcified and the clotting time at 37 C noted

Table 5 shows that dilution shortens the clotting time

#### 4 Effect of concentration of calcium on Howell's time

Lozner, Joliffe, and Taylor<sup>15</sup> state that the addition of calcium to whole blood obtained from their patient produced a shortening of the clotting time We have not observed any effect in the case reported here from the addition of concentrations of calcium chloride between 0.05N and 0.0125N

#### 5 Effect of storage of plasma on Howell's time

When the patient's citrated plasma was kept at room temperature for 48 hours, the Howell's time was not modified provided the plasma was devoid of platelets

#### 6 Effect of normal plasma and fractions of normal plasma on the clotting defect of the patient's plasma

TABLE 5 — *Effect of Dilution on Howell's Time*

Date	Howell's time of undiluted plasma (minutes)	Howell's time of diluted plasma (0.2 cc plasma + 0.8 cc physiological saline) (Minutes)
7-25-46	360	40
3- 1-46	360	40
11- 7-46	300	90
11-14-46	300	90

We have used normal human plasma and one of the plasma fractions obtained by Cohn and his coworkers in the department of Physical Chemistry of Harvard Medical School \* The plasma fraction used was Fraction I of Cohn, which in addition to fibrinogen contains most of the antihemophilic activity of normal plasma Two types of fraction I were used One from normal human plasma, and the other from hemophilic blood plasma † These fractions were obtained as a dry powder 40 mg of which was dissolved in 2 cc physiologic saline at pH 7.5

Table 6 shows that neither type of fraction I in the quantities used in this experiment had any effect on the clotting defect of the patient's plasma

#### 7 Quick's prothrombin time of the patient's plasma

This was determined by means of brain's thromboplastin the prothrombin time of the patient's plasma determined by this method was 20 seconds, very near the prothrombin time of the normal control (table 7) However, with smaller amounts of thromboplastin there appears a widening divergence between the patient's plasma and the normal control (table 7)

\* Obtained through the courtesy of Dr. E. J. Cohn

† Obtained through the courtesy of Dr. F. H. L. Taylor

## 8 Effect of thrombin on citrated plasma absence of immediate antithrombin

The preparation used was obtained from the Roussel's laboratories as a dry powder, 50 mg of which assayed 12 Iowa units. Fifty milligrams was dissolved in 2 cc of physiologic saline. At all dilutions used (table 8), it was impossible to detect a significant immediate antithrombic action in the patient's plasma.

TABLE 6—Effect of Normal Plasma, Fraction I from Normal Plasma and Fraction I from Hemophilic Plasma on Clotting Time of Platelet's Plasma Reagents in cc

Patient's plasma	Normal plasma	Fraction I normal	Fraction I hemophilia	Clotting Time*
				minutes
0.1	1	0	0	40
0.1	0	1	0	120
0.1	0	0	1	120

\* After addition of 1.1 cc of  $\text{CaCl}_2$  to each tube at 37 C

TABLE 7—Effect of Dilution of Thromboplastin on Prothrombin Time (0.1 cc Citrated Plasma + 0.1 cc Solution of Thromboplastin + 0.1 cc  $\text{CaCl}_2$ , 0.025 N)

Dilution of thromboplastin	Prothrombin time (seconds) of	
	Patient's plasma	Normal plasma
1/1	20	18
1/10	32	26
1/50	63	50
1/100	84	62
1/200	127	83
1/500	180	116
1/1000	390	140

TABLE 8—Effect of Thrombin on Citrated Plasma (0.25 cc Plasma + 0.5 of Thrombin Solution at 37 C)

Dilution of thrombin	Clotting time of normal human citrated plasma	Clotting time of patient's citrated plasma
	seconds	seconds
1/1	11.5	11
1/2	19.5	20
1/4	36	37
1/16	100	106
1/32	228	270

## 9 Progressive antithrombin

Normal serum or plasma contain an antithrombin which is called 'progressive' because it inactivates thrombin when incubated with thrombin. Table 9 shows that the patient's plasma did not inactivate thrombin more actively than did normal plasma and did not therefore contain more progressive antithrombin than did normal plasma.

## 10 Antifibrinolytic activity of the plasma

It was decided to test this activity in the patient's plasma because there are similarities between the trypsin inhibitor from soya bean, and the anticoagulant

present in the patient's plasma Both substances are ant clotting but not anti-thrombic They both are thermostable It became necessary to investigate whether the patient's plasma exhibited enhanced antiproteolytic or antifibrinolytic activity

TABLE 9—*Effect of Incubation of Patient's Plasma with Thrombin Thrombin Added to Defibrinated Plasma, the Mixture Incubated 15 Minutes at 37 C and the Clotting Time on Fibrinogen Measured at 37 C Defibrination Was Done by Heating at 56 C for 5 Minutes*

Reagents in cc thrombin	Normal plasma	Patient's plasma	Saline	Fibrinogen Solution	Clotting time
					<i>seconds</i>
0.4	0	0	0.1	0.5	9
0.4	0.1	0	0	0.5	36
0.4	0	0.1	0	0.5	42

TABLE 10

A Fibrinolysin 1 cc + Citrated Plasma 0.05 cc + Thrombin 0.1 cc Reading Done after Incubation at 37 C for 18 Hours

Dilutions of fibrinolysin	Lysis	
	Patient's plasma	Normal plasma
1/1	+	+
1/2	+	0
1/4	0	0
1/8	0	0
1/16	0	0
1/32	0	0
1/64	0	0

B Fibrinolysin 0.2 cc Plasma 0.2 cc Diluted with 0.2 per cent Solution of Fibrinogen Reading Done after Incubation at 37 C for 18 Hours

Dilutions of plasma	Lysis	
	Patient's plasma	Normal Plasma
1/1	0	0
1/2	0	0
1/4	0	0
1/8	+	0
1/16	+	+
1/32	+	+
1/64	+	+
1/128	+	+
1/256	+	+

As a source of fibrinolysin, we used a preparation of chloroform plasma (Nolf-Tagnon) The fibrinolysin preparation was added to citrated plasma and a clot was obtained by the addition of thrombin The disappearance of the clot was taken as a measure of the intensity of the fibrinolysis

11 Finally we have observed that the quantity of anticoagulant agent in the patient's plasma increased from August to November 1946. This is shown by the fact that in August a mixture of 1 volume of citrated normal plasma and 1 volume of citrated patient's plasma, after addition of calcium, clotted in 50 to 60 minutes, while in November, in order to obtain the same clotting time, it was necessary to add 4 volumes of normal plasma to one volume of the patient's plasma. It is difficult to say at the present, whether this increase was the result of the transfusion of blood, as was the case in the patient described by Munro.<sup>17</sup>

### DISCUSSION

The patient discussed in this communication was clinically a hemophiliac. This diagnosis was based on a prolonged clotting time, a normal bleeding time, and the occurrence of hemorrhagic episodes and joint hemorrhages. The disease started at the age of 18 months. However, certain aspects of the case were not in agreement with the diagnosis of hemophilia among which the absence of a family history of the disease and the lack of effect of blood transfusions. Also, atypical were the lack of effect of the addition of either normal plasma or fraction I of Cohn on the clotting time in vitro of the patient's blood. Finally, and contrary to what occurs in true hemophilia, the blood or plasma from the patient had a definite anticlotting effect on normal blood or plasma.

There is little doubt that the blood of this patient contained a circulating anticoagulant. Munro<sup>4, 5</sup> has reported a similar occurrence in a patient classified as hemophilic. In Munro's case, the appearance of the anticoagulant followed the use of repeated blood transfusions. In the case reported here, such an etiology seems unlikely in view of the fact that the patient had received one single transfusion before the time that the circulating anticoagulant was observed in his blood. This single transfusion was given him when he was a child. Later transfusions, given after the circulating coagulant was observed, had no effect on the clotting time or, if they had any effect, it was in the direction of *prolonging* the clotting time, although this prolonging effect was difficult to demonstrate since the blood, before transfusion, clotted in the very long time of 6 hours at 37° C. We have given this patient one transfusion only because of the detrimental effect of this treatment in this type of cases, as described by Munro.<sup>17</sup>

The anticoagulant present in the plasma of this patient is thermostable. We were able to heat the plasma at 65° C. for 30 minutes without causing the disappearance of the anticlotting activity. At ice box temperature the activity is maintained for 8 days at least. At room temperature, the activity decreases after 48 hours. The anticoagulant appears not to be identical with heparin since we have observed no neutralizing effect from the addition of toluidine blue. Furthermore, quantities of protamine between 0.001 mg. and 0.1 mg. for 1 cc. of plasma did not affect the clotting time.

The anticoagulant was not an antithrombin and did not appear to be antithrombolytic. The prothrombin time determined by the method of Quick was not significantly altered.

Concerning the mechanism of action of the anticoagulant, one can say that this

principle acts on the first phase of the coagulation phenomenon since we have shown that it is not antithrombin. It would appear therefore that it opposes the transformation of prothrombin into thrombin.

The anticoagulant could conceivably oppose this transformation by one of three possible mechanisms: (1) by an action on calcium—that this was not the case is affected by the fact that the blood calcium was normal and that varying the amount of calcium did not correct the prolonged clotting time, (2) by an action on prothrombin—this seems unlikely since the prothrombin time was normal, on the other hand we have mixed 0.1 cc of the patient's plasma with 1 cc of prothrombin poor plasma (obtained from a patient treated with dicumarol) containing 33 per cent of the normal level of prothrombin. The clotting time of the mixture (38–48 minutes) was found to be similar to that of a control (38–45 minutes), (3) by an action on thromboplastin—this is rendered unlikely by the fact that small quantities of thromboplastin added to the patient's plasma corrected the clotting defect quite completely. The antithromboplastin described by Tocantins,<sup>6</sup> in contrast to the anticoagulant described here, is destroyed by heating at 50 C.

Other hypotheses should be briefly considered. According to Lenggenhager,<sup>7</sup> the thromboplastin originating from platelets exists as a prothromboplastin and is activated by contact with foreign surfaces and calcium, while the thromboplastin derived from cellular material exists in an active form. According to this hypothesis, the anticoagulant described here could conceivably work by opposing the activation of the prothromboplastin.

According to Nolf,<sup>8</sup> Howell,<sup>9</sup> Patek and Stetson,<sup>10</sup> Patek and Taylor,<sup>11</sup> Feissly,<sup>12</sup> Bendien and Van Creveld,<sup>13</sup> blood plasma contains an essential factor of blood coagulation distinct from prothrombin. This factor exists in plasma as an inactive precursor. It is thermolabile and is bound to the englobulin fraction of plasma. It is called antihemophilic factor, globulin substance, plasma thromboplastin, etc.

According to Frederica,<sup>14</sup> and, independently, to Ferguson,<sup>18</sup> the plasma tryptase is probably the active principle which in association with calcium and the platelets activates prothrombin. This author thinks that component A of prothrombin, described by Quick,<sup>1</sup> is identical with the plasma tryptase.

A definite explanation of this mechanism of action of the anticoagulant described here must await a better understanding of the mechanism of normal coagulation itself. Our impression is that this anticoagulant acts on either the mechanism of activation of prothromboplastin, or the antihemophilic substance, or the plasma tryptase.

An interesting question is whether our anticoagulant is identical with the anti-clotting substances described in 3 different publications.<sup>15 16 4</sup> Certain common features suggest an identity: these are the thermostability, lack of antithrombin action, lack of action on the prothrombin time.

The substance described by Munro<sup>4 17</sup> appears to be a  $\gamma$  globulin.

#### SUMMARY

A new observation of a hemorrhagic diathesis associated with the presence of an anticoagulant in the circulating blood is reported here. The patient was a 21

year old male, appearing by clinical evaluation to have hemophilia, but without a family history of hemophilia. The blood and plasma were strongly ant clotting and had a very long clotting time. The clotting time of recalcified citrated plasma was greatly delayed by removing the platelets. Freezing and thawing of platelet-rich plasma resulted in a marked shortening of the clotting time. Dilution of the plasma shortened the clotting time, while the addition of calcium and storage of the plasma had no effect.

The prolonged clotting time was not corrected by the addition of normal plasma or plasma fractions having antihemophilic activity. The prothrombin time was nearly normal. Small quantities of thromboplastin were very effective in shortening the clotting time. The anticoagulant had no antithrombin activity. The "progressive" antithrombin and antifibrinolysin of the patient's plasma were normal.

The anticoagulant acts during the first phase of coagulation by inhibiting an (plasma) activator of prothrombin. It appears to be identical with the anticoagulant described in three previous publications from the United States.

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# THE RELATIONSHIP BETWEEN HEPARIN DOSAGE AND CLOTTING TIME

By L. B. JAKES, M. A., PH. D., AND ANN G. RICKER, M. A.

THE GREAT interest of Dr. Minot in all branches of hematology has always included an appreciation of the problems involving the anticoagulants. The recent development of anticoagulant therapy has increased this interest. We therefore are pleased to present as an indication of our esteem a recent study of factors affecting the action of heparin *in vivo*. We believe that a study of this nature is of value in leading to an understanding of the various physiologic factors involved in this action and hence to an appreciation of the principles involved in its administration.

Heparin when used experimentally or clinically is usually controlled by the determination of the prolonged clotting time produced. It was shown in a previous paper<sup>1</sup> that the value of the clotting time produced will depend on both the coagulant power of the blood and the ability of the body to remove heparin from the circulation. Crafoord<sup>2</sup> reported that the blood became more resistant to the action of heparin after operation, suggesting an increase in coagulability. Following this lead, De Takats,<sup>3</sup> Waugh and Ruddick,<sup>4</sup> and Whittaker<sup>5</sup> have suggested tests of blood coagulability based on the resistance of the blood to the anticoagulant power of heparin. In a continuation of the previous study, further observations of the effect of heparin dosage on the clotting time in the dog have been made. These observations give a clearer concept of the factors involved both in the use of heparin as a test of the clotting function of the blood and also in its administration.

## METHODS

Dogs of 10 to 20 Kg. body weight were used. The experiments were conducted on trained, unanesthetized animals unless otherwise indicated. Unless otherwise stated, blood samples were taken by venous puncture from superficial veins through the skin. Care was taken to ensure that the sample was taken expeditiously without trauma. The syringe and needle were first rinsed with saline and emptied of air, saline being left in the needle. Normally, 2 cc. of blood were taken and the first 0.5 cc. and last 0.5 cc. of blood were discarded. If larger quantities of blood were required the same technic was used, larger amounts of the first and last of the sample being discarded. Clotting times were determined at 37°C. in the coagulometer described by Murray, Jakes, Perrett and Best.<sup>6</sup>

We are indebted to the Connaught Medical Research Laboratories, University of Toronto, for a generous supply of heparin. This was received as a solution of 1000 Connaught units/cc. (9.0 mg. per cc.) of the sodium salt of beef heparin. The unit is the anticoagulant activity of 1/100 mg. of the crystalline barium salt as prepared by the method of Charles and Scott.<sup>7</sup> This unit is as closely as one can ascertain, the same as the provisional International Unit<sup>8</sup> which is expected to replace it, and also the original unit used by Howell. In these studies the heparin was injected intravenously.

## CLOTTING TIME RESPONSE TO HEPARIN IN VITRO

When the clotting time is determined with varying amounts of heparin added to the blood *in vitro*, a curve such as that of *a* in figure 1 is obtained. The practical

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significance of this curve is that a certain level of heparin is required before there is any significant effect on clotting time, whereas increases in heparin concentration beyond this value result in the clotting time being very markedly increased for slight increases in heparin concentration. Several empirical equations have been suggested for this curve.<sup>1 9 10</sup> However, a simple procedure is to plot the data on semilogarithmic paper, whereupon a straight line is obtained. In figure 1 this has been done to give curve *b*. The same procedure has previously been recommended for the relation between thrombin concentration and clotting time<sup>11</sup> and appears to be a consequence of the determination of clotting times, since a similar

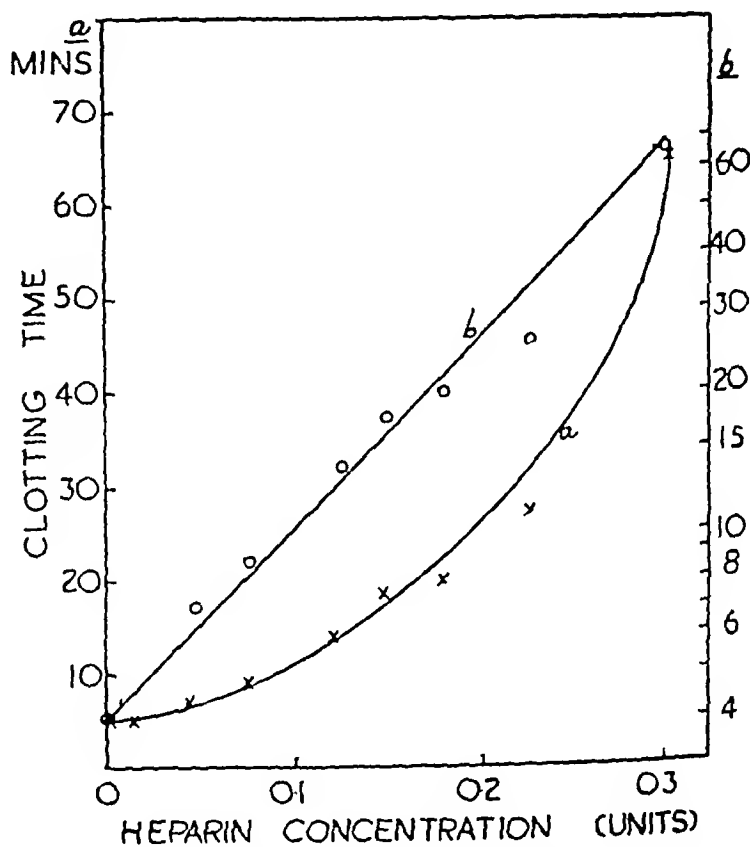


FIG. 1. Effect of heparin concentration in vitro on clotting time. Blood samples taken from the exposed femoral vein and 1 cc. added to the quantities of heparin shown.

logarithmic relation can be obtained for many coagulant and anticoagulant substances.

It would appear of practical importance to have some single value to express the sensitivity of a blood sample to the anticoagulant action of heparin. This is easily achieved by taking the slope of the straight line obtained on semilog paper. This gives us the "heparin sensitivity value," and is calculated from  $\frac{\log T_2 - \log T_1}{c_2 - c_1}$ , where  $T_2, T_1$  = clotting times and  $c_2, c_1$  = heparin concentrations at two points on the line. Since the coagulometer used measures clotting times to the nearest minute only, the greatest error was on the normal clotting time. Hence, the line

was drawn to give the best fit for all the points, without necessarily passing through the normal value. Typical results obtained in a series of 15 dogs are shown in table 1. The clotting times as those in figure 1 were determined on blood removed from an exposed femoral vein. It can be seen that the range for the heparin sensitivity values for the animals was from 0.50 to 48.8. These extreme values were for only two animals, representing the hyperreactors and hyporeactors of De Takats. The values for 50 per cent of the animals were in the range 2.3 to 3.2, while 5 (33 per cent) were moderate hyperreactors giving values of 3.8 to 7.6. The difference between different animals with regard to their sensitivity to heparin is a relatively permanent difference. Determinations on the same animal, 10 days apart, shown in table 2, give sensitivity values agreeing to within 10 per cent, while the

TABLE 1—*Sensitivity of the Blood to the Action of Heparin in Dogs\**

Dog	Normal Cl T † Mins	Heparin sensitivity Value	Cl T with heparin	
			0.1 units Mins	0.5 units Min
A-19	3.6	24.2	α	α
A-20	2.0	50	2.2	3.6
A-21	2.0	48.8	α	α
A-22	1.0	5.48	3.6	α
A-26	4.0	4.12	10.5	α
A-31	1.0	3.22	2.1	41
A-34	2.5	2.80	4.7	63
A-35	2.0	7.28	10.4	α
A-36	3.0	2.84	5.8	78
A-37	1.1	4.30	4.7	α
A-38	2.9	2.31	5.0	42
A-39	3.7	2.65	8.7	α
A-42	0.9	2.38	1.7	15
A-43	1.0	2.76	2.0	25
A-44	2.0	2.46	3.6	34
Median	2.0	2.8	4.7	78

\* Blood samples taken from exposed vein

† Cl T—Clotting Time α—>90'

difference between the average normal dog and the hyperreactors is one of several hundred per cent.

Another method of measuring sensitivity to heparin, although by no means as informative, is to report the clotting time for a single dose of heparin. This has been done in table 1 for 0.1 and 0.5 units of heparin/cc. Also reported is the normal or control clotting time, i.e., the clotting time without added heparin. The value of the clotting time for 0.1 units of heparin was from 1.7 minutes (almost the same as the normal) to over 24 hours. The median value was 4.7 minutes. Another way of giving the values is the concentration of heparin required to double the normal clotting time. This was 0.008 units for dog A-21, and 0.6 units for dog A-20, the median value for the series was 0.11 units. There appeared to be no correlation be-

tween the normal clotting time and sensitivity to heparin. Both hyper- and hyporeactors were found with normal clotting times longer than the average value.

The sensitivity of blood to the anticoagulant action of heparin depends (1) on the individual from whom the blood is taken, and (2) on the technic used both for removal of the blood and for determination of the clotting time. In the experiments reported in table 1, the dogs were under an anesthetic and the blood samples were taken from the exposed femoral vein. Direct experiments showed that this procedure increased the sensitivity of the blood sample to heparin. In a later series, reported in table 2, the blood was taken by the usual venipuncture procedure through the skin. The decrease in heparin sensitivity is shown by the decrease in the sensitivity value to the level 0.8–1.4, the median being 1.03, or a clotting time of 12.1 minutes for 0.5 units. Hyper- and hyporeactors were not used for this series. The series again demonstrates the individual variation even in a series of animals selected with regard to their response to heparin. Likewise, there is no indication from the normal clotting time of their response to heparin.

The above demonstrates one factor which can change the sensitivity of the blood sample for heparin. Other factors which can change this are the use of saline to moisten the syringe, removal of the needle before transferring the blood to the tube for the determination, surface coatings, etc. Finally the temperature at which the determination of clotting time is conducted markedly affects the sensitivity of the sample. Heparin shows least anticoagulant activity at 20–25°C, its activity increasing above and below this temperature range.

While the great variation in the response of the clotting time to heparin due to changes in technic of venipuncture and determination of clotting times might suggest that the use of clotting times should be discarded as far as heparin is concerned, actually the value of such determinations is greatly increased when these factors are appreciated and utilized. As can be seen from figure 1, the relation between the clotting time and heparin concentration is such that measurable changes in clotting time can be obtained only over a rather narrow range of concentrations, a considerable handicap in experimental studies on heparin. However, by suitably modifying the method, it is possible for any desired range of heparin concentration within reason to be made measurable. With proper control of these technical factors, satisfactory reproducible results are obtainable with any given method.

In using heparin both experimentally and clinically, it would appear advisable to administer it on a weight or potency basis (i.e., in milligrams or units per kilogram) rather than on the basis of an arbitrary clotting time. Since the same dosage is not necessarily required for all uses, the dosage can be modified to the particular use. It follows from the above discussion that the method for the determination of clotting time, used to determine the duration of heparin action in the circulation as discussed below, may be suitably modified to give measurable clotting times at this dosage level. While the dosage for a particular purpose may be established for the average individual, it may be advisable to modify it for those individuals whose blood proves to be either highly sensitive or relatively insensitive to the anticoagulant action of heparin.

## THE CLOTTING TIME RESPONSE TO HEPARIN IN VIVO

As first reported by Howell,<sup>12</sup> a single intravenous injection of heparin is followed by a rapid rise in the clotting time, and then by a more gradual but still rapid decrease to the normal value. As seen from the relationship between heparin concentration and clotting time in vitro, the rapid changes in clotting time at the higher values are due to the nature of the relationship between heparin concentration and clotting time. Hence they do not reflect actual changes in heparin concentration in the blood, and in order to use the clotting time to follow changes in heparin concentration in the blood it is advisable to plot the data, as with the in vitro curves, on semilog paper. When this is done, as in figure 2, it can be seen that

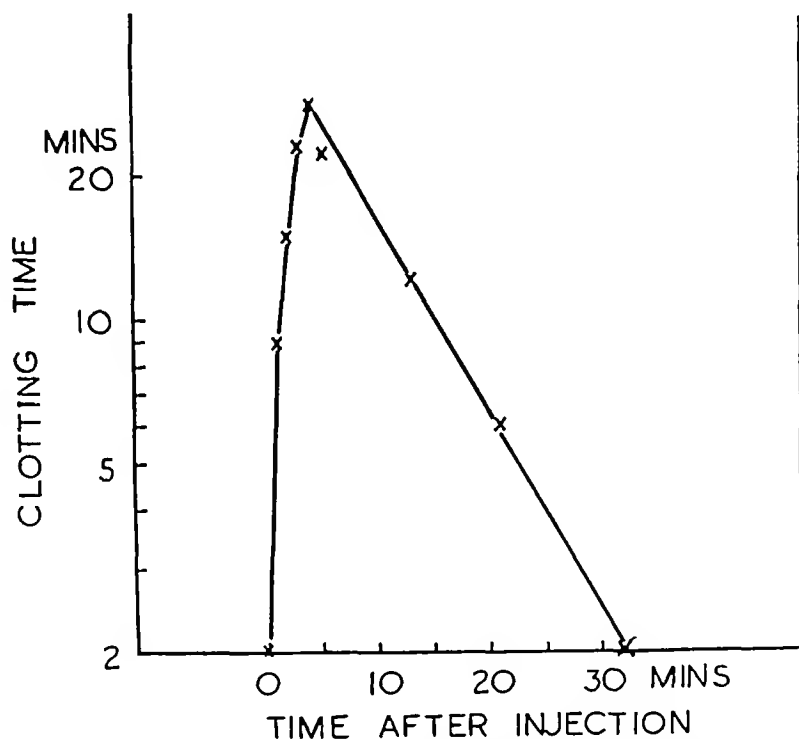


FIG. 2. Effect of heparin in vivo on clotting time. Single intravenous injection of  $30 \mu/\text{Kg}$  given at zero time.

the anticoagulant effect of heparin disappears from the circulation in a relatively linear fashion, at the concentration given by this dosage of heparin.

In studying the clotting time response of the animal to injected heparin (fig. 2) three different values are of interest. These are (1) the time required for the clotting time to reach its peak value, (2) the maximum clotting time reached, and (3) the time required for the clotting time to return to normal, or the duration of the hypocoagulable effect. These three values will be considered separately.

*Time required for the clotting time to reach its peak value.* In figure 2, with a dose of 30 units  $\text{Kg}$ , the maximum clotting time was reached in four minutes. With a circulation time of 20 seconds, one would expect that the heparin would have exerted its maximum effect within one minute after the injection. Actually, with larger

doses of 100 units/Kg, one usually finds that a ten to fifteen minute period is necessary for the clotting time to reach the peak value. It is evident from this that the delay in developing the hypocoagulability of the blood following the injection of heparin is not due simply to the time required for mixing with the blood. As discussed later, an explanation of this delay has been found. It also should be pointed out that this effect is observed only with doses of heparin which give clotting times that are still measurable. With very large doses (1000 units/Kg) complete incoagulability develops immediately.

De Takats<sup>1</sup> has reported that the clotting time response to injected heparin is biphasic. He noted a rise to the maximum within one minute of injection. This was followed by an immediate return to the normal value which was then followed by the rise in clotting time usually observed. No evidence of such a biphasic response is observable in the data reported in figure 2 nor have we observed any evidence of this in a series of dogs in which samples were taken for clotting times every minute after injection. De Takats' results were obtained on man. However, it is difficult to deduce any rational basis for such a phenomenon, since, as shown below, there is essential, even though not complete, quantitative agreement between the clotting times obtained when the heparin is mixed with the blood *in vivo* and *in vitro*.

*The maximum clotting time.* It was concluded previously that the maximum clotting time obtained on the injection of heparin was the same as if a corresponding amount of heparin were mixed with the blood sample *in vitro*. In the present investigation a more exact test of this hypothesis was conducted, using the present methods. In each case the effect of heparin concentration on clotting time was first determined *in vitro* by adding blood samples to various concentrations of heparin. The results were then plotted on semilog paper to give a straight line and the slope of the line (heparin sensitivity value) is reported in the table. Thirty or 100 units/Kg were then injected and the clotting time determined at frequent intervals, as in figure 2. In table 2 is shown the maximum clotting times obtained for the injections. The clotting time for the same concentration of heparin added to the blood *in vitro* was determined from the *in vitro* experiment, the blood volume being taken as  $1/11$ th of the body weight. This is likewise reported as the peak clotting time (*in vitro*). It can be seen that of fifteen injections the clotting time *in vivo* was always much greater than that obtained *in vitro*, the value obtained from mixing *in vivo* being three to ten-fold that *in vitro*.

The only difference between the two series is that in the one case the heparin mixed in the body is in contact with the blood for the five to ten minutes required for the clotting time to rise to the maximum. Quick<sup>13</sup> has reported that heparin shows an enhanced activity on incubation with an oxalated plasma system. In order to test the effect on heparin activity of incubation with blood, 10 cc of blood was drawn into 10 cc of sodium oxalate solution. One cubic centimeter of oxalated blood was then added to each of a series of tubes containing 0, 0.1, 0.25 and 0.5 units of heparin and the tubes were allowed to stand thirty minutes at 25°C. The tubes were then placed in the coagulometer, equivalent calcium was added and the clotting time was recorded. A control series of tubes was set up similarly, but the

heparin was added at the end of the thirty-minute period before the addition of calcium

The results for several animals are shown in experiments 1 and 2, of table 3. It is evident that after incubation with heparin the clotting times of the samples are much longer. In order to avoid the decalcification, the experiment was also conducted in another way. It has previously been shown by Jaques, Fidler, Feldstedt and Macdonald<sup>14</sup> that coating glassware and needles with the silicone "Dri-Film"

TABLE 2.—*Effect of Heparin on Clotting Time in Dogs\**

Dog	Expt	In Vitro			In Vivo		
		Cl T † (no heparin) Mins	Sensitivity Value	Peak Cl T Mins	Peak Cl T Mins	Duration Mins	Dose/Time
Heparin—30 μ/Kg							
A	29	2 8	1 34	6 9	26	32	90
C	30	2 6	1 18	10 2	28	32	90
E	34	3 0	1 68	9 6	>60	36	84
F	36	2 1	0 85	3 8	16	30	1 0
E	39	3 7	0 94	7 3	14	29	1 0
D	42	3 0	0 78	5 1	19	40	75
D	48	4 6	1 37	13 0	34	30	1 0
E	50	4 1	1 03	8 2	>90	25	1 2
D	52	4 5	0 96	8 7	22	24	1 3
E	56	3 7	1 24	8 7	42	39	76
D	57	4 1	0 99	8 2	24	30	1 0
Average		3 5	1 14	8 15	34	32	0 93
Heparin—100 μ/Kg							
C	33	3 5	1 34	77	>210	190	53
E	43	—	—	—	73	115	87
D	49	4 6	—	36	90	105	95
E	50	4 1	1 03	44	>240	125	80
E	54	2 0	2 30	400	>240	112	89
D	55	4 5	—	—	>120	95	1 05
Average		3 7				124	0 80

\* Blood sample by venipuncture through the skin

† Cl T—Clotting Time

gives a surface which is apparently inert to the clotting system and that, provided the blood sample is taken in such a way as to prevent contamination with damaged tissue, such blood does not clot for several hours. Five cubic centimeters of blood were removed from a superficial vein with a silicone-coated syringe and needle. The first and final 0.5 cc of blood were discarded and 1 cc portions of blood added to varying amounts of heparin in silicone-treated tubes. These were allowed to stand 10 minutes at 37 C. The samples were then transferred with an untreated syringe

and needle to the coagulometer and the clotting times were recorded. The values are shown in experiments 3, 4, 5 and 6, table 3, opposite I. The control tubes in experiments 3 and 4 were set up identically and the heparinized blood then transferred to the coagulometer immediately without incubation. In experiments 5 and 6 the control blood was added to the silicone tubes, incubated ten minutes, and then added to the heparin in the coagulometer tubes.

It can be seen that, irrespective of changes in the clotting time of the blood following incubation (zero heparin), there is a marked increase in the clotting time of the heparinized samples. This result was obtained consistently, with marked differences in technic and in the coagulability of the blood of the individual animal. Evidently a certain period of time is required for heparin to combine with the com-

TABLE 3—*Effect of Incubation of Blood with Heparin on Clotting Time*

Heparin Units		Clotting Time, Mins			
		0	1	25	50
1	C	1	6	45	95
	I	1	13	105	143
2	C	1	3	6	20
	I	1	4	25	40
3	C	2	8	18	70
	I	<1	7	25	90
4	C	2	10	28	84
	I	<1	11	58	106
5	C	1	4	9	18
	I	1	5	20	41
6	C	1	8	18	90
	I	1	7	43	140

C—Control or unincubated series, I—Series with blood incubated with heparin.  
Experiments 1 and 2 with oxalated blood, 3, 4, 5 and 6 with fresh blood in silicone.

ponents of the clotting system before it can exert its maximum anticoagulant effect. It appears reasonable to attribute to this factor both the discrepancy between the clotting times obtained on mixing the heparin with the blood *in vitro* and *in vivo*, and also the lag in the rise of the clotting time to its maximum. A great excess of heparin would accelerate the combination of heparin with the necessary factors of the clotting system, and thus cause the disappearance of the lag. As reported above, it was actually found experimentally, that the lag phase disappeared with large doses of heparin.

*Duration of the hypocoagulability after heparin injections.* The duration of hypocoagulability represents the time during which the anticoagulant activity of heparin remains in the circulation. Reed<sup>15</sup> reported that this time was directly proportional

to the dosage. In the previous study by Jaques<sup>1</sup> this was confirmed, for when the dosage was divided by the time, the ratio obtained was approximately constant and was independent of the dosage level and of variations in individual animals. The value obtained was 2.0 units/Kg/min. Shown in table 2 is the duration of the hypocoagulability for a series of animals receiving injections of 30 and 100 units/Kg. In the present series, likewise, the ratio is constant. The agreement is in fact considerably better than previously, but the average value is 0.9 units/Kg/min. Such a discrepancy is much too great to be due to experimental errors and merits further consideration.

Several factors were different in the two studies. First, crude heparin (15 units/mg) was used in the first series, while all later experiments were conducted with pure heparin. Direct comparison of crude and pure heparin, however, showed no difference in the clotting time response elicited. Secondly, a series of doses (from 31 to 667 units/Kg) were used in the previous experiments, while only two dosage levels (30 and 100 units/Kg) were used in the present series. It is significant that in the previous study the values of the ratio for the 31 and 113 unit doses were 1.2 and 1.6 respectively, compared with values of 1.9 to 3.4 (average 2.5) for doses of 200 units/Kg and over. This result suggests that the value of the ratio changes with the dosage level of heparin. Thirdly, the clotting times were previously determined by the Lee and White method at 25°C, with blood samples taken from the superficial vein through the skin, and it was found that this method failed to detect heparin in concentrations in the blood lower than 0.3 units/cc. On the other hand, the present method detects concentrations of heparin as low as 0.10 units. Hence, at the time when the clotting time was normal by the first method, heparin was still present to give a prolonged clotting time by the second method, thus decreasing the ratio. This factor appears to increase in importance with increasing sensitivity of the clotting time method used to detect the heparin. Thus Jaques, Charles and Best<sup>16</sup> reported data for the injection of 35 units/Kg in dogs. The clotting time was determined with the coagulometer, but blood samples were obtained from the exposed femoral vein, thus further increasing the sensitivity. In their data, the hypocoagulability lasted from 40 to 80 minutes, again providing a further discrepancy on comparison with the data reported in table 2. However, this effect on this ratio of increasing the sensitivity of the test method for heparin could be predicted from the results reported by Jaques,<sup>1</sup> of the effect on the clotting time of continuous injections of heparin. He found that the rate of disappearance of heparin from the blood at blood levels below 1 unit per cc was a function of the blood level, so that increasing the sensitivity of the test would result in the detection of heparin in the blood for a relatively much longer time. He likewise reported that at blood levels greater than about 1.5 units per cc, the heparin disappeared from the blood at a relatively constant rate. This, of course, would result in an apparent proportionality between dosage and duration of hypocoagulability, if the time during which blood levels below 1.5 units per cc could be detected were small in comparison with the total period of hypocoagulability.

It is evident then that the constancy of the ratio of dosage to the duration of



hypocoagulability is more apparent than real. However, the ratio may have some value when used in a strictly empirical manner.

### THE COMBINED HEPARIN SENSITIVITY TEST

Waugh and Ruddick<sup>4</sup> have recently described a test for changes in the coagulability of the blood based on the effect of heparin on the clotting time when added *in vitro*. De Takats' test,<sup>5</sup> on the other hand, is based on the response when the heparin is injected. Such tests are of value both as a possible indication of suspected thrombotic tendencies and as an indication of the dosage of heparin required. The *in vivo* test as suggested by De Takats is essentially a 'heparin tolerance curve'. As is evident from the previous discussion, this tolerance curve is established by two separate groups of factors. The first is the coagulability of the blood itself. This will determine both the value of the maximum clotting time and also at what level of heparin concentration in the blood it will be no longer possible to determine the residual heparin present by means of clotting time determinations. The second factor consists of those processes whereby the heparin activity disappears from the circulation.

It would appear of value, in studies on heparin at least, to use a test which distinguishes between and tests both of these factors. This can be done by determining the response of the individual to heparin added to the blood both *in vitro* and *in vivo*. By thus using both types of response we can obtain a maximum amount of information regarding these systems. Unfortunately, the incubation effect described above, which is effective *in vivo*, forbids the direct quantitative application *in vivo* of the data obtained in the '*in vitro*' test. Variations in the rate of reaction of heparin with the clotting factors (the so-called incubation effect) can probably be determined by inspection of the initial portion of the *in vivo* curve, however. Finally, the value of both tests, and the information obtained from them, can be greatly increased by suitable plotting on semilog paper to give linear relationships. In this way it is possible to obtain the maximum information from the few points which practical considerations allow one to determine.

In our laboratory the test is actually conducted as follows: 0, 0.2, 0.5 and 1.0 units of heparin in 0.3 cc. of saline are taken in tubes in the coagulometer. Five cubic centimeters of blood is drawn, as described under *Methods*, and 1.0 cc. is added to each tube, the first and last portions of blood being discarded. The clotting times of these samples are determined, establishing the *in vitro* curve. Thirty units

Kg. of heparin are then injected intravenously and the clotting time determined at five to ten minute intervals. This is usually repeated with a 100 units Kg. dose, to give two *in vivo* curves. Typical results are shown in figures 3 and 4. It is our impression that the first blood sample taken from an individual animal has a longer clotting time than do subsequent samples. We therefore prefer to use this for other purposes or discard it when conducting clotting studies.

*The effect of anesthesia on the clotting time response to heparin.* It has long been known that anesthetics affect the clotting time. It was necessary, therefore, to examine their effect on the test. A series of dogs, after repeated testing, were anesthetized and the response again determined. The effect of a barbiturate (sodium pentobar-

bital) is shown in figure 3. This anesthetic was given intravenously. It can be seen that pentobarbital definitely increased the clotting time response. This effect was relatively greatest, however, on the normal clotting time (without heparin) indicating a decrease in the coagulability of the clotting system. This was evidently relatively independent of the action of heparin, since the two lines converged. It can also be seen that the difference in the response *in vivo* after this anesthetic can be explained on the basis of this change in the coagulability of the blood.

Four dogs were tested for their sensitivity to heparin during anesthesia with ether by inhalation with the open mask method. The results of these experiments were somewhat contradictory and did not show the reproducibility in the response

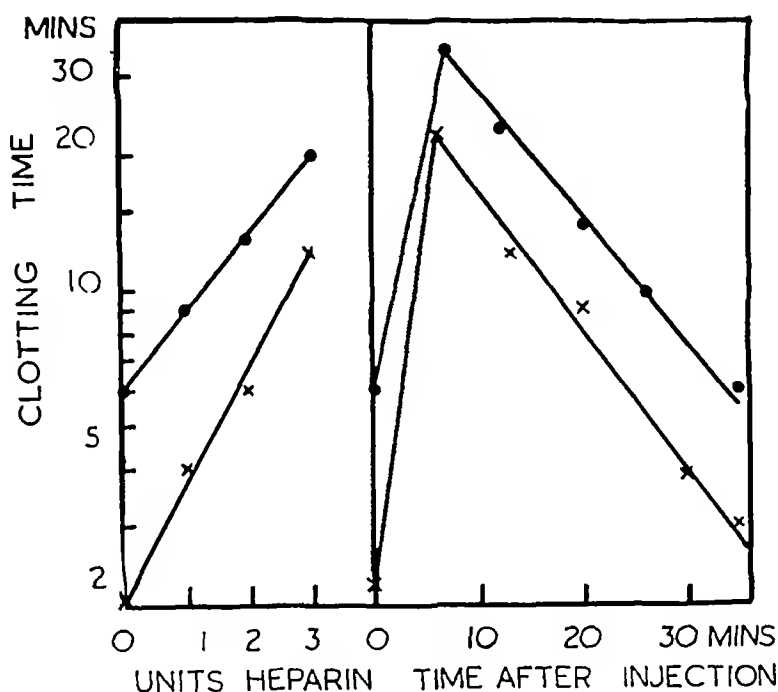


FIG 3 Effect of anesthesia with sodium pentobarbital on the heparin sensitivity test. Left-hand figure is for the *in vitro* test, right-hand figure for the *in vivo* test. —x—x— normal unanesthetized animal. —●—●— same animal under pentobarbital anesthesia.

seen with the barbiturate anesthesia and the nephrectomy experiments to be discussed later. Two dogs showed no change in clotting time with ether, one showed an increase, the other a decrease. Three of the four dogs showed a definite increase in sensitivity to heparin *in vitro*. In all four dogs there was fairly close agreement in the response *in vivo* with and without the anesthetic. It has been suggested that the effect of ether anesthesia on clotting is due to the liberation of adrenaline. This would explain the variability in response as judged by the heparin test. Two dogs were anesthetized with urethane. There was no change in clotting time of the animals but a definite decrease in heparin sensitivity. There was no change in the *in vivo* response.

It is evident from the above that the test is affected by anesthetics. The var-

ability under ether indicates that caution must be exercised in applying the test with this anesthetic. However, the use of the test is possible following anesthesia with pentobarbital.

*The effect of treatment with india ink on heparin sensitivity.* Various investigators have suggested a relationship between heparin and the reticulo-endothelial system. Godlowski<sup>17</sup> originally reported that the injection of heparin resulted in a blockage of the activity of the reticulo-endothelial system as judged by the uptake of india ink. Rigdon<sup>18</sup> has recently completely failed to confirm this. On the other hand, Knisely, Bloch, and Warner<sup>19</sup> have confirmed it microscopically. Volkert<sup>20</sup> observed in rabbits that the injection of india ink caused the disappearance of 20 per cent of the antithrombin, being the fraction in the blood considered by him to be heparin. Also it prevented the rise in this fraction which he normally observed after the injection of protein. The effect of india ink, he concluded, was due to an effect on the red cells whereby they could bind heparin.

TABLE 4—Effect of India Ink Injections on the Heparin Tests

Exp	Dog	Time after india ink injection	In vitro test		In vivo test Peak clotting time	Duration
			Clotting Time	Slope		
			min		min	min
35	E	preinjection	3 0	1 64	>60	36
		10'	5 4	0 37	—	—
		3 hours 40'	—	—	10	43
		6 days	3 7	0 96	14	29
56	E	preinjection	2 0	2 30	42	39
		4 hrs	3 7	1 24	12	23
37	F	preinjection	2 1	0 85	16	30
		4 hrs	2 8	0 96	4	20

A series of dogs was standardized, and 5 cc of a 5 per cent suspension of india ink (Higgins) was given intravenously. The response of the animals to heparin was then again determined. The tests were conducted at various times after the india ink. Immediately after the injection the blood was hypercoagulable to such an extent that it was not possible to conduct the tests. In only one case (experiment 35) was it possible to obtain a satisfactory heparin curve immediately after the injection. The india ink had disappeared from the plasma ten minutes after the injection as judged by the color of the plasma. The most interesting and consistent effect of india ink was observed three to four hours after the injection. Examples of this are shown in table 4. At this time, a marked flattening of the response to heparin *in vivo* was observed as shown by the marked decrease in the peak clotting time. The *in vitro* test showed some decrease in the sensitivity to heparin. However, this did not always appear to be responsible for the marked effect on the *in vivo* curve. The duration of the *in vivo* test was decreased in some cases but not consistently with the decrease in peak clotting time. The normal clotting time of the animal was lengthened. The effect lasted partially as long as six days after the injection of the ink.

The results resemble those of Volkert, suggesting an interference in the reaction of heparin with the clotting system rather than either a change in the coagulability (which would be demonstrated by the *in vitro* test) or a change in the ability of the body to inactivate heparin (which would be demonstrated by the duration of the hypocoagulability *in vivo*). Rather, there is an increase in the blood of nonspecific factors which interfere with heparin action. Since blockade of the reticulo-endothelial system with india ink does not increase the duration of hypocoagulability after heparin injections, the reticulo-endothelial system is probably not involved in the disappearance of heparin from the circulation.

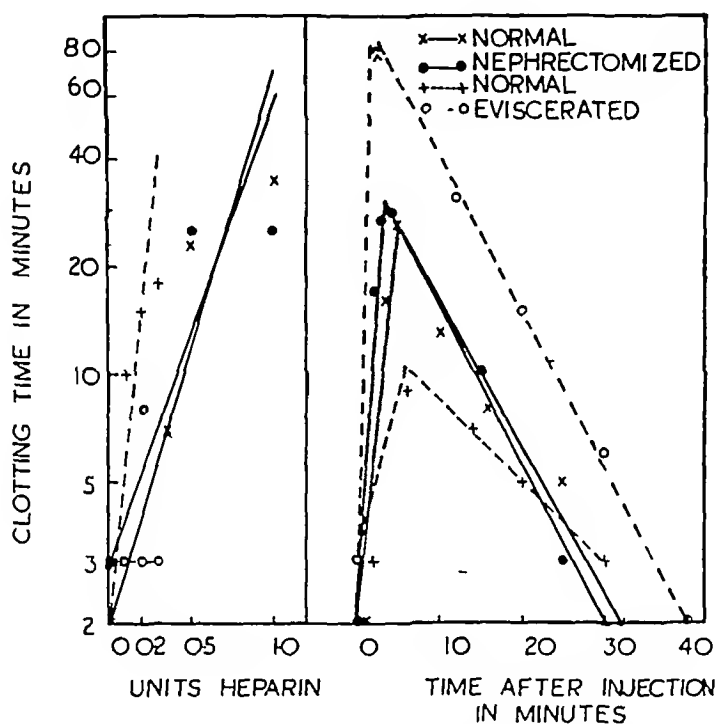


FIG. 4. Effect of nephrectomy and evisceration on the response to heparin. Left-hand figure, *in vitro*, right-hand figure, *in vivo*. x—x animal under pentobarbital anesthesia before operation, ●—● same animal after removal of the kidneys + - - - + animal under pentobarbital anesthesia, ○ - - - ○ same animal after evisceration.

*Effect of nephrectomy and evisceration on heparin sensitivity.* As shown in figure 2, the anticoagulant action of heparin disappears rapidly from the circulation. Howell suggested that heparin was excreted by the kidneys, and evidence in favour of this view has been obtained by Wilander<sup>21</sup> and Copley and Schnedorf<sup>22</sup>. However, Jaques<sup>1</sup> and Reinert and Winterstein<sup>23</sup> did not detect any excretion in the dog or in man. As a more direct test of this problem, both kidneys were removed from four dogs under pentobarbital anesthesia. These animals had been carefully standardized on heparin and the heparin sensitivity was again tested immediately after the operation while the animals were still under the anesthetic. The results of one experiment are shown in figure 4. It can be seen that there is no change in the response

to heparin either *in vitro* or *in vivo*. While the points of the *in vitro* curve do not lie on the straight line as closely as is usually the case, there is no significant difference between the values before and after operation. One animal showed an increase in clotting time and one showed a decrease, after the operation. In these experiments, the curves after operation were simply displaced by a corresponding amount, indicating that there had been no change in sensitivity to heparin *in vitro* or *in vivo*.

Since the kidneys do not appear to be directly responsible for the rapid disappearance of hypocoagulability after heparin injections, the gastrointestinal tract was similarly investigated. Three dogs were carefully standardized for the heparin test under pentobarbital anesthetic. The gastrointestinal tract and related organs, including the spleen, were then removed. Since removal of the liver is followed by a rapid fall in prothrombin, introducing a further difficulty in interpreting the test, care was taken not to damage the liver or hepatic artery. After completion of the operation, a good pulse was observed in the hepatic artery. The response of the animal to heparin was determined one hour and three hours after the operation. Glucose was given postoperatively to two animals, but not to the third. No difference was observed in the response of the three animals.

The results obtained in one animal are shown in figure 4. After the operation the animal's blood was hypercoagulable, as shown by the lack of response to heparin added *in vitro*. The response *in vivo* was also changed. A much greater peak clotting time was obtained. This was to be expected, since in removing the viscera we also removed a large proportion of the effective blood volume, so that the heparin concentration of the 30 unit/Kg dose was proportionately much greater when calculated as units/cc of blood. However, the clotting time returned to normal in 35 minutes, compared with 30 minutes before operation. It is evident that the gastrointestinal tract likewise is not responsible for the disappearance of heparin from the circulation.

#### SUMMARY

1. The relationship between clotting time and heparin dosage has been studied in the dog.

2. On the addition of heparin to blood *in vitro*, a linear relation is found between heparin dosage and the logarithm of the clotting time obtained. The sensitivity of the blood sample to the action of added heparin is influenced both by the individual (coagulability of the blood before withdrawal) and by the technics of withdrawal and of determination of the clotting time. It is indicated that alterations in the latter may be used to extend the range of measurable hypocoagulability due to heparin. Incubation of heparin with blood for ten minutes increases its anticoagulant effect.

3. When moderate doses of heparin are injected intravenously, five to fifteen minutes are required for the clotting time to reach a maximum. No evidence of a biphasic response was obtained. The maximum clotting time obtained is greater than it is with the same amount of heparin added to the blood *in vitro*, due to the effect of incubation of heparin with blood on its anticoagulant activity. The in-

terval required for the clotting time to return to normal is quite short, and with a given dosage is constant with different animals. Factors influencing the relation between duration of hypocoagulability and dosage are discussed.

4. A test has been devised to determine the sensitivity of the animal to the anti-coagulant action of heparin. The clotting time response to certain concentrations of heparin added to the blood *in vitro* is determined. A fixed dose of heparin is then injected intravenously and the clotting time response is again determined. The response *in vitro* measures the sensitivity of the clotting system to heparin, while the *in vivo* response, when interpreted in the light of the *in vitro* response, measures the ability of the body to remove heparin from the circulation.

5. By means of this test, it has been determined that anesthesia with pentobarbital decreased the coagulability of the blood, urethane had no effect on coagulability, while the effect of ether was variable. The injection of india ink and evisceration caused a hypercoagulability, while removal of the kidneys had little effect.

6. When the sensitivity of the blood to the anticoagulant action of heparin was tested during these procedures, pentobarbital and nephrectomy had no effect, ether caused an increase in sensitivity, urethane a decrease. The injection of india ink and also evisceration markedly decreased the sensitivity of the blood to the anti-coagulant action of heparin.

7. Anesthesia with pentobarbital, ether or urethane, the injection of india ink, removal of the kidneys, or removal of the gastrointestinal tract, had no effect on the duration of heparin action in the body.

#### ACKNOWLEDGMENT

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# BLOOD

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### RESPONSE OF TROPICAL SPRUE TO VITAMIN B<sub>12</sub>

By TOM D SPIES, M D , AND RAMON M SUAREZ, M D

THROUGHOUT the world the macrocytic anemias are of great interest to physicians Sprue, perhaps the most common syndrome in this group, has a geographic distribution restricted to India, Ceylon, the Malay States, the Philippine Islands and, to a lesser extent, the Southern United States and the countries bordering or near the Caribbean sea After Minot and Murphy<sup>1</sup> demonstrated that liver was effective in promoting blood regeneration in persons with Addisonian pernicious anemia, it was found that tropical sprue could be treated with liver and liver extracts with some success In recent years two pure chemical compounds, pteroylglutamic acid (folic acid) and 5-methyl uracil (thymine), have been shown to be effective in the treatment of sprue<sup>2-3</sup> Still a third chemical substance, vitamin B<sub>12</sub>, has recently been isolated which is infinitely more potent per unit of weight than liver extract, folic acid, or thymine, but it has not been synthesized, and at the present time it is available only in small amounts for experimental use This compound, shown by Shorb<sup>4</sup> to be a growth factor for *Lactobacillus lactis* Dorner and to bear an almost linear relationship to the potency of concentrated liver extract, was isolated from liver by Rickes, Brink, Koniuszy, and Folkers<sup>5</sup> West<sup>6</sup> has demonstrated its positive hemopoietic action in three cases of pernicious anemia Spies, Stone, and Aramburu<sup>7</sup> have reported both a hematologic and a clinical response in two cases of pernicious anemia, two cases of nutritional macrocytic anemia, and one case of nontropical sprue Spies, Garcia Lopez, Milanes, Lopez Toca, and Culver<sup>8</sup> have reported a hematologic and clinical response following its administration to two cases of tropical sprue The present communication is concerned with the response of five cases of tropical sprue in Puerto Rico to vitamin B<sub>12</sub>

These patients were selected for study using the following criteria (1) The patient must have macrocytic anemia as determined by Wintrobe indices (2) The bone marrow must show the typical megaloblastic type of maturation arrest seen in macrocytic deficiency anemias (3) the erythrocyte counts must be below 2.5 million (4) The patient must be untreated, or must not have been treated recently enough to interfere in any way with the evaluation of the vitamin B<sub>12</sub> (5) He must have persistently low reticulocyte counts during the preliminary period of

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This study was aided by a grant from the Martha Leland Sherwin Memorial Fund

The vitamin B<sub>12</sub> was furnished by Dr. Augustus Gibson, Merck & Company, Inc.



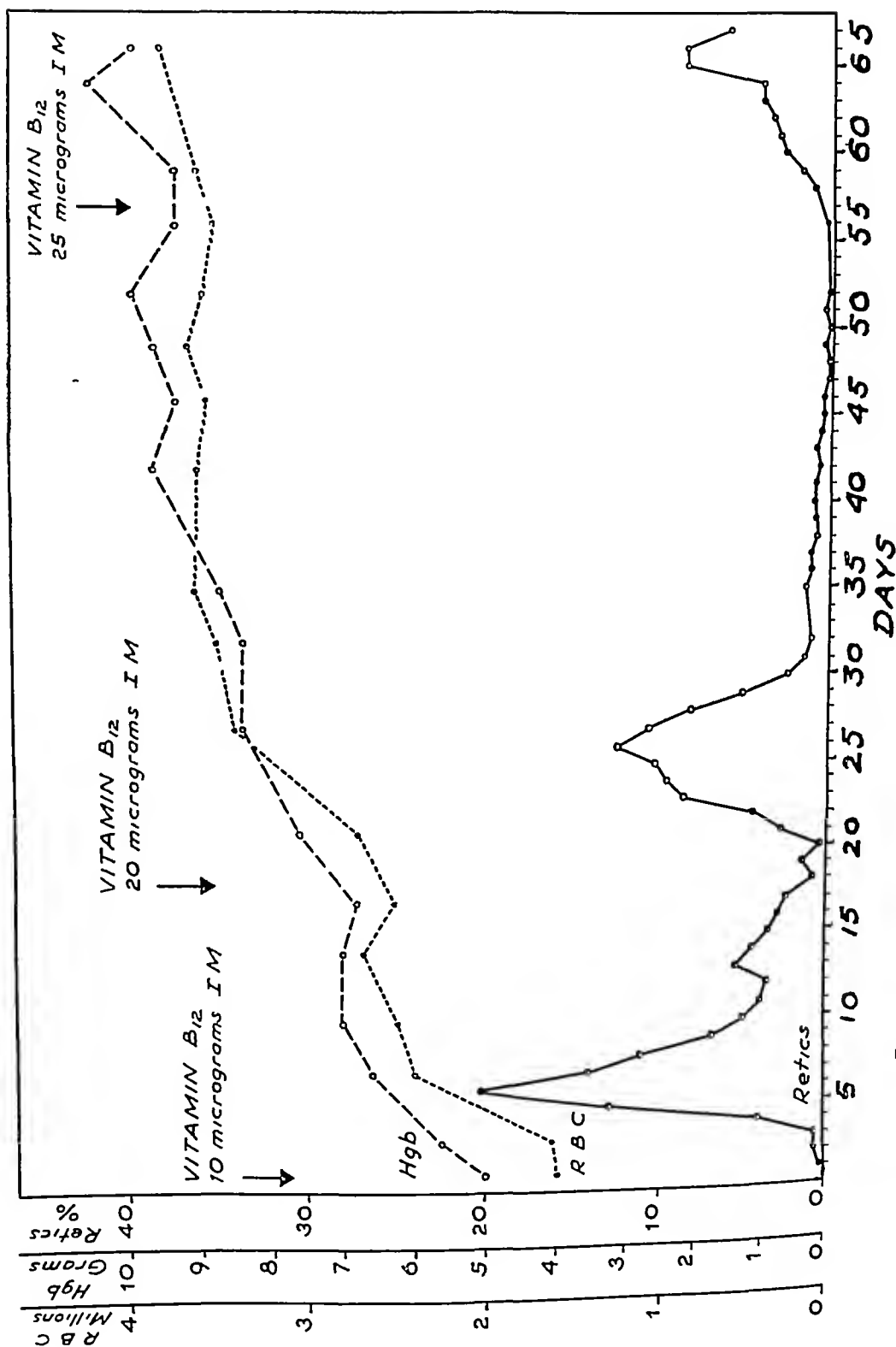
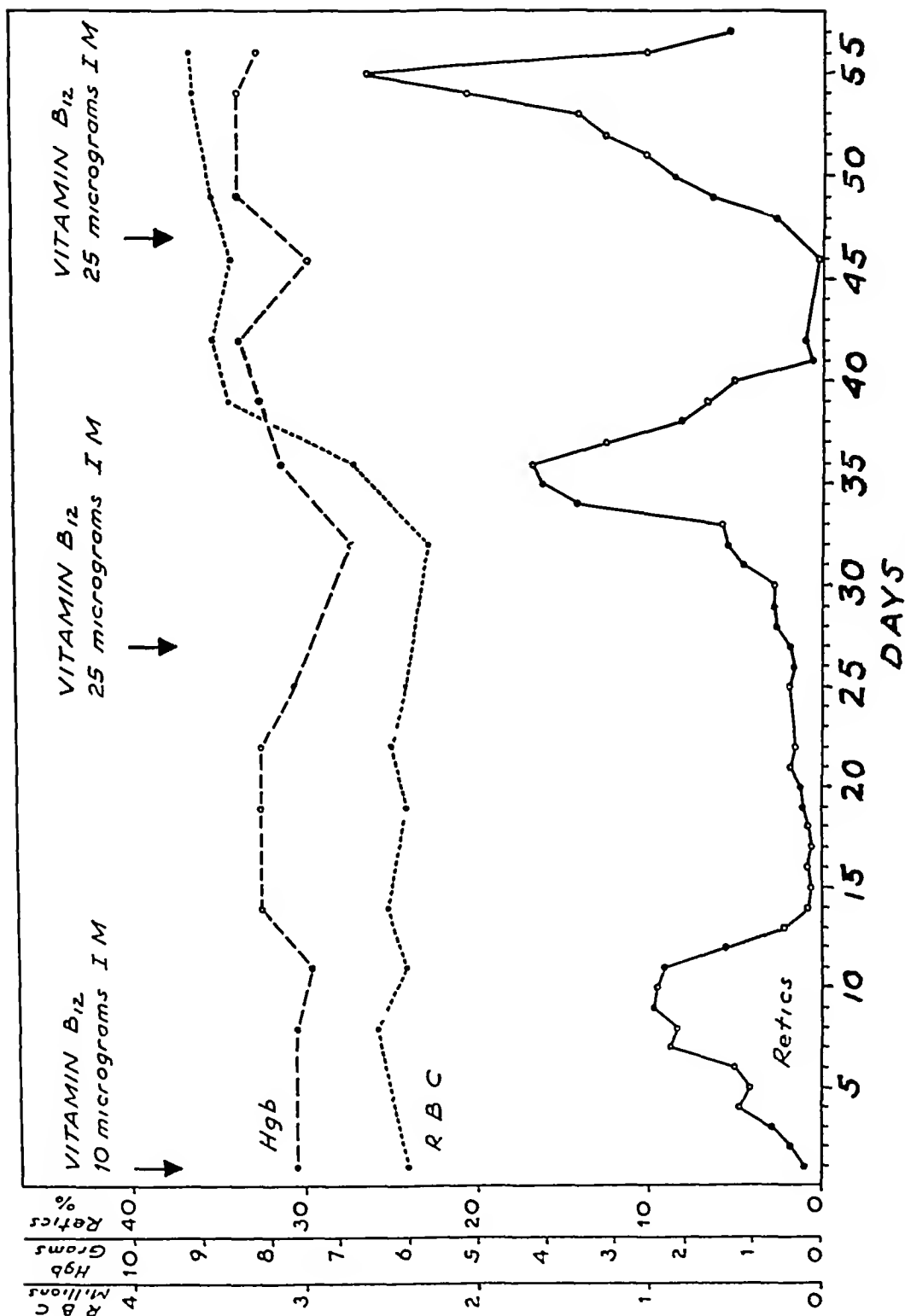


FIG. 1. HEMOPOIETIC RESPONSE OF PATIENT (E. R.) WITH TROPICAL SPRUE TO VITAMIN B<sub>12</sub>

FIG. 2. HEMOPOIETIC RESPONSE OF PATIENT (D R) WITH TROPICAL SPURIE TO VITAMIN B<sub>12</sub>

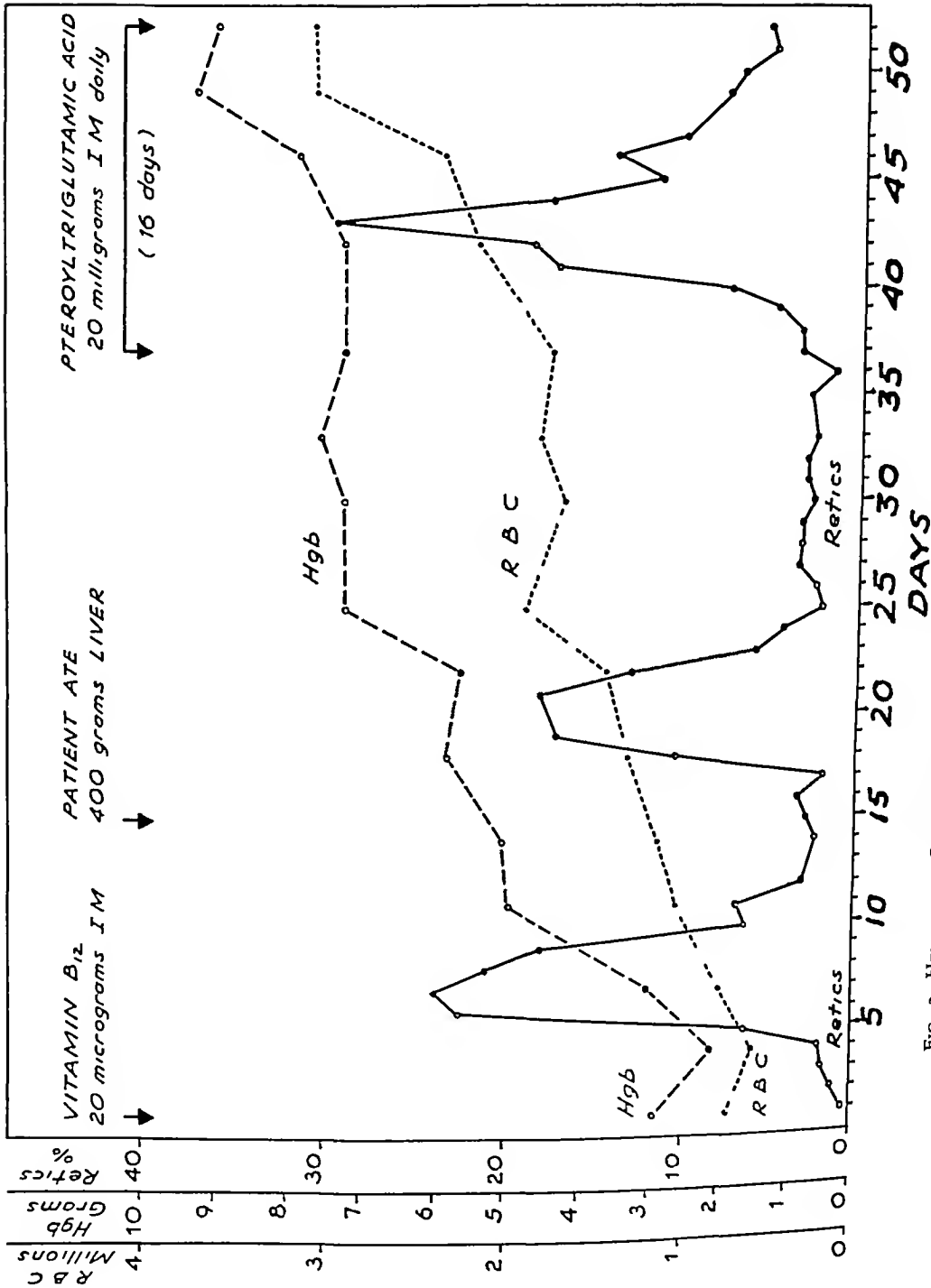
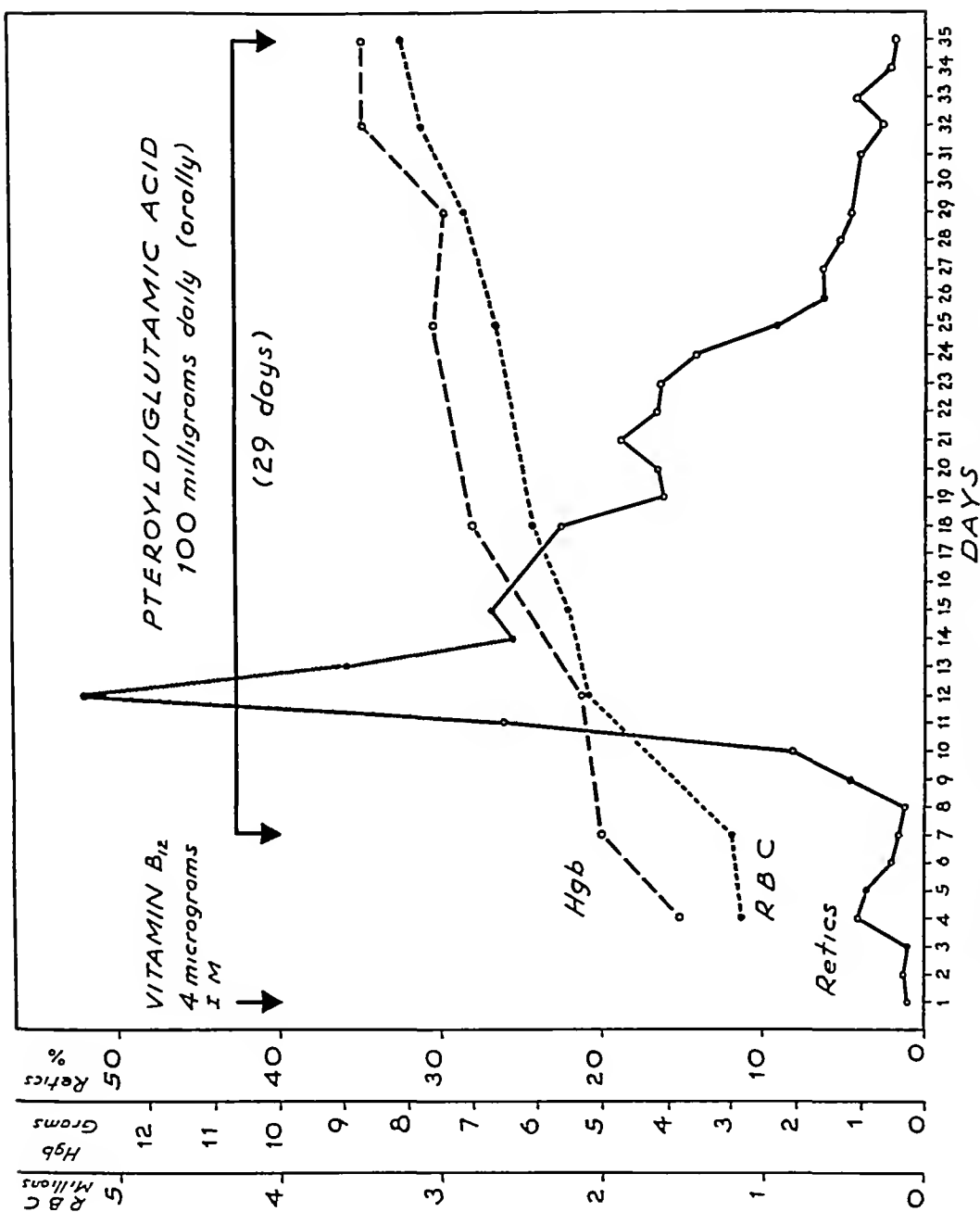
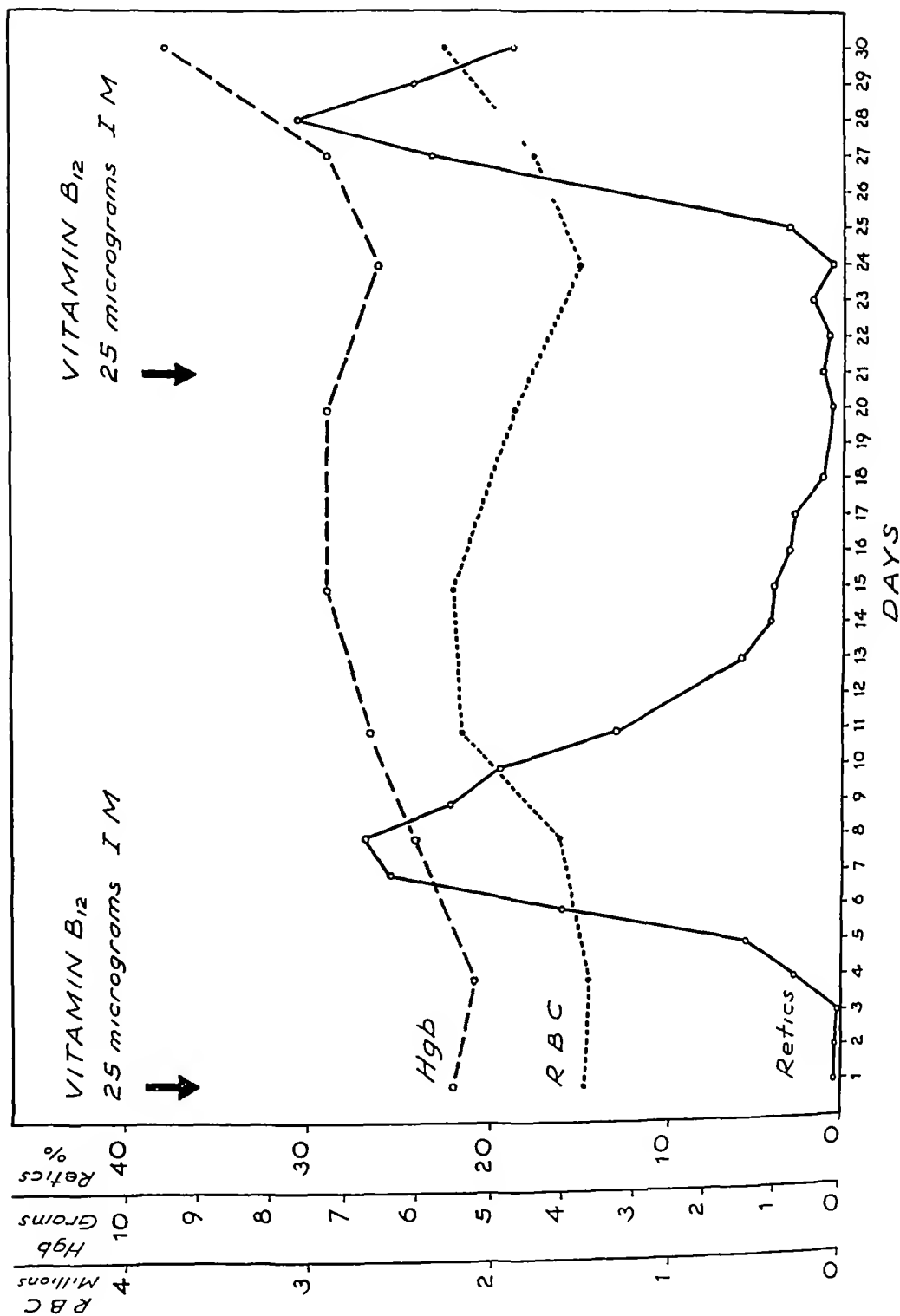


FIG 3 HEMOPOIETIC RESPONSE OF PATIENT (A S) WITH TROPICAL SPRUE TO VITAMIN B<sub>12</sub>

FIG. 4. HEMOPOIETIC RESPONSE OF PATIENT (M C) WITH TROPICAL SPUR TO VITAMIN B<sub>12</sub>

FIG 5 HEMOPOIETIC RESPONSE OF PATIENT (J G) WITH TROPICAL SPRUE TO VITAMIN B<sub>12</sub>

observation (6) He must have alimentary tract symptoms consistent with diagnosis of tropical sprue

Hematocrit studies were made using pipets certified by the United States Bureau of Standards The hemoglobin content was determined by means of the Photovolt photoelectric hemoglobinometer, calibrated so that 14.5 grams was equivalent to 100 per cent The reticulocytes were counted in dry preparations of brilliant cresyl blue counterstained with Wright's stain Platelets were enumerated in the counting chamber used for red blood cells by means of a fresh solution of sodium citrate

Sternal bone marrow was obtained by aspiration prior to treatment and again near the peak of reticulocytosis

Gastric analyses were performed in each case

On admission the patients were given the 'preliminary' sprue diet previously described<sup>9</sup> and were maintained on this diet throughout the period of study After the baseline studies were completed, the five patients selected were treated and the results are shown in figures 1, 2, 3, 4, and 5, which illustrate the hematologic response in each case

#### OBSERVATIONS

Reticulocytosis occurred in each case and usually began around the fourth day, being followed by erythrocytosis and hemoglobin production Coincidental with the reticulocytosis, clinical improvement occurred in cases 1, 2, 3, and 5 This improvement was characterized in each patient by a gain of strength and a great increase in appetite and feeling of well-being As can be seen by a glance at the figures, the red blood cells and the amount of hemoglobin increased after the peak of the reticulocytosis, but in no instance did these patients respond maximally

In case 4, receiving 4 micrograms of vitamin B<sub>12</sub>, it is questionable whether the response which occurred was due to the very small amount of vitamin B<sub>12</sub> administered This case responded in a characteristic way to large amounts of pteroyldiglutamic acid (fig 4)

Case 3, who responded both clinically and hematologically to a single dose of 20 micrograms of vitamin B<sub>12</sub>, ate 400 grams of liver fifteen days later A spectacular improvement occurred (fig 3), this figure also shows the subsequent response to the administration of pteroyltriglutamic acid

It is of considerable interest that after a second injection of vitamin B<sub>12</sub> in case 5, she again responded well A similar response was noted following additional injections of vitamin B<sub>12</sub> in cases 1 and 2 Not only was there an additional increase in blood values, but additional clinical improvement occurred and the alimentary tract function tended to return toward normal as the bowel movements decreased in number and the stools became darker and better formed

#### SUMMARY AND CONCLUSIONS

These findings show that the administration of vitamin B<sub>12</sub> to patients with tropical sprue was followed by general clinical and hematologic improvement provided the dosage was adequate A single dose of 4 micrograms administered in case 4

produced little or no change. The larger dosage of 10-25 micrograms administered in the other cases was accompanied by striking increase in strength and vigor and a decrease in the diarrhea, however, in no instance was a maximal dose given and these patients quickly tended to relapse clinically and hematologically. They could be relieved promptly again either by another injection of vitamin B<sub>12</sub> or by a compound of folic acid (The conjugated compounds of folic acid used in these cases were used for experimental purposes, and they produced the same hematologic response as that of folic acid per se.) Case 3, who had an excellent hematologic response after eating one serving of 400 grams of liver, is regarded as especially significant in that it suggests that, as powerful as vitamin B<sub>12</sub> is as a therapeutic agent, it is more effective when given with liver. It is especially noteworthy that cases 1 and 2, who had three injections of vitamin B<sub>12</sub>, have had steady clinical and hematologic improvement. The reader should have in mind that a single injection of approximately 100 micrograms of vitamin B<sub>12</sub> probably would be needed to produce a full hematologic response in persons so ill. This tentative appraisal would suggest that this therapeutic compound, per unit of weight, is more effective in treating human disease than any compound that yet has been used.

#### ACKNOWLEDGMENT

We are very much indebted to others who have aided us in selecting cases and in observing results. Especially we wish to thank Dr. F. Hernandez-Morales, Dr. Hector Marchand, Miss Clemencia Benitez Gautier, and Miss Sara Torres.

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# ADULT GAUCHER'S DISEASE, WITH SPECIAL REFERENCE TO THE VARIATIONS IN ITS CLINICAL COURSE AND THE VALUE OF STERNAL PUNCTURE AS AN AID TO ITS DIAGNOSIS

By J. GROEN, M.D., AND A. H. GARRER, M.D.

GAUCHER'S disease is a rare, often familial disease, distinguished by the presence of characteristic cells in the organs of the reticulo-endothelial system (spleen, liver, lymph glands, bone marrow). The protoplasm of these cells has a typical cytologic appearance due to the presence of a special lipid, the so-called Gaucher substance or kersin.

The increase in our knowledge of the underlying pathologic process has gradually facilitated the possibility of a clinical diagnosis during life. Gaucher<sup>6</sup> described the disease in 1882 as an "idiopathic hypertrophy of the spleen, without leukemia," but he mentioned in his paper that in the course of the disease the liver also became enlarged. Another sign, the swelling of lymph glands, has proved of little diagnostic value as the swollen glands are usually situated inside the body. An important observation, of help in diagnosis, may be a peculiar yellow pigmentation which develops in some of the cases on the face and in the conjunctivae in the form of wedge-shaped pingueculae. Bloem, Groen and Postma<sup>2</sup> and Kveim<sup>11, 12</sup> drew attention to the presence of a characteristic pigmentation that occurred fairly commonly on the lower legs in their patients suffering Gaucher's disease. If present, this pigmentation is almost pathognomonic of this condition. Bloem, Groen and Postma<sup>2</sup> described a few other minor signs that might be of additional help in the diagnosis, viz., the presence of a peculiar malar flush and the occurrence of myopia.

Examination of the blood may yield further useful information: a hypochromic anemia with leukopenia and thrombopenia are usually present, the cholesterol content of the blood is normal or very often, low. However, this hematologic syndrome occurs also in other conditions associated with splenomegaly so that its diagnostic importance is not decisive. The majority of patients exhibit some degree of hemorrhagic diathesis.

X-ray examination has supplied us with another means of supporting a clinical diagnosis of Gaucher's disease. Some of the early investigators, most notably Brill, Mandlebaum and Libman,<sup>3</sup> demonstrated that Gaucher cells accumulate in various parts of the bones where they ultimately produce macroscopic areas of bone destruction. Further experience has shown that these bone lesions, although they may occur almost anywhere, show a predilection for certain areas. The head and neck of the femur are often affected, which gives rise to a typical x-ray picture. The lower ends of the femora often contain so many Gaucher cells that they become swollen and present a shape similar to an Erlenmeyer flask.

Even in the presence of these clinical signs, however, a diagnosis of Gaucher's

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Publication of this report was delayed by the war and the occupation of The Netherlands.



disease could very often not be made with absolute certainty. The introduction of the sternal puncture was therefore a great advance. It is the purpose of this paper to present some cases of Gaucher's disease with special reference to the importance of sternal puncture as an aid to the diagnosis. At the same time this will give us an opportunity to describe the clinical features which these cases presented. Some of the cases have been described by others,<sup>4 13 14, 15 26</sup> and by one of us,<sup>2</sup> years ago and it seems appropriate to present their development since that time.

## CASE REPORTS

### CASE I

Mrs. E. R. B., born in 1890, a Jewish married woman, was first seen in the out-patient department on January 5, 1938.

*History.* The patient had always been a weak girl. When she was 13 years old she noticed a lump in the left side of the abdomen which grew slowly during the years that followed. A tendency to bleed manifested itself at an early age, later on bleeding was especially troublesome after tooth extractions and during menstruation. In 1922, when she was 32 years old, a splenectomy was performed by Dr. Pimentel. On microscopic examination this spleen was found to contain the typical Gaucher cells.\*

After the operation her condition remained fairly satisfactory until 3 years before admission, when she began to suffer from palpitation, dyspnea on exertion, and a persistent cough. Occasionally she would bring up some sputum, sometimes blood-stained. The urine had become scarce and darker than it had formerly been. She was thirsty but had not noticed any swelling of the feet.

The family history did not reveal other cases of a similar nature. The patient's father died of cancer of the rectum, her mother of apoplexy. Both died in a hospital, no enlargement of the spleen or liver had been found in these individuals. The patient's husband, two sisters, one brother and her two children, 25 and 19 years old respectively, were also examined by us. None of them showed any of the clinical signs of Gaucher's disease.

*Physical examination.* The patient appeared weak and in a rather poor nutritional state. There was marked cyanosis of nails, ears, lips and cheeks. The entire skin had a somewhat yellowish tinge. Both conjunctivae showed typical pingueculae. There was bilateral myopia.

The heart was markedly enlarged toward the left, the apex-beat was visible in the anterior axillary line. The right border extended 1 cm. outside the right sternal line. All heart sounds were loud, the first mitral and the second pulmonary sounds were especially accentuated. There was normal percussion over both chest fields anteriorly, but posteriorly there was dullness on the right side, reaching from the spine of the 7th thoracic vertebra downward. On auscultation, fine crepitant rales could be heard over both lungs, over the right lower lobe the breath sounds were diminished. The abdomen showed a large surgical scar. The liver was enlarged and firm and reached downward to the umbilicus.

The right lower leg showed pigmentation of the skin due to the application of heat. The typical Gaucher pigmentation was not present. The blood pressure was normal.

The urine contained some albumin ( $\frac{1}{4}$  per cent), an excess of urobilin and some casts. Morphologic blood studies showed hemoglobin, 110 per cent, erythrocytes, 5,530,000, and leukocytes, 11,200. The cholesterol, lipid phosphorus, and lipid nitrogen content of the blood plasma were normal.

The x-ray picture showed a diffuse decalcification of the bones but no localized areas of destruction. The heart appeared displaced and/or dilated to the left. There was fluid in the right hemithorax. The pulmonary artery was abnormally prominent.

Sternal puncture revealed many typical Gaucher cells.

The clinical diagnosis was Gaucher's disease with a tentative diagnosis of mitral stenosis or cor pulmonale due to increased resistance of blood flow in the smaller circulation. It was supposed that the obstruction in the smaller circulation was due to an accumulation of Gaucher cells in the lungs. It was

\* The specimen was demonstrated by M. Elshout in a meeting of the Netherlands Pathological Society.<sup>4</sup>

significant that in this case (where the spleen had been removed) leukocytosis was present instead of the usual leukopenia. The thrombopenia, however, persisted after splenectomy.

The condition gradually became worse. On August 10, 1938, the patient was admitted to the hospital. This time there was fluid on both sides of the chest. During her stay in the hospital the patient had repeated attacks of severe dyspnea with extreme cyanosis. She died suddenly during one of these attacks. Her age at death was 48 years.

Permission for postmortem examination could not be obtained, but punctures of the liver and the sternum were performed immediately after death. In both, Gaucher cells were found. The number of these cells in the bone marrow appeared to be far greater than in the liver.

#### CASE 2

Mr. Fa, a Jewish lawyer, born in 1901, of German origin, was first seen on October 27, 1937.

*History.* The patient had no complaints at all until 1932, when he began to suffer from pain in the right leg, which started in the buttock and radiated toward the foot. The pain disappeared after a period of rest in bed. In 1933, he had similar pains in both legs, which disappeared spontaneously after ten days. In May, 1934, the same pain, which he considered to be sciatica, recurred in the left leg, this time it stayed there. All movements in the left hip joint became increasingly difficult, it was impossible for him to cross his legs when sitting. Now and then he felt distinct crepitation in the left hip joint on movement.

In October, 1934, he also experienced pain in the right side of his back. The local doctor suspected gall stones and sent him to a roentgenologist. The night before the x-ray examination, the patient took tetraiodo-phenolphthaleine and the following morning he went to the hospital without breakfast. He cannot remember what happened to him that morning, but apparently he arrived at the hospital in a dreamlike state. The x-ray picture was made, but the patient's memory returned only when, after coming home at 11:00, he took his breakfast. He then told his family that he had to go to the hospital for the x-ray picture, he had no idea that he had already been there. No gall stones were found on this occasion. Subsequently he has had similar states of mental confusion. Finally, they occurred almost every morning between awakening and breakfast. After breakfast the symptoms disappeared. A sister of the patient noticed that during these trances his pupils became enlarged and that the patient perspired and trembled all over. At this time the patient was examined in Düsseldorf where the diagnosis of cerebral tumor was made. Lumbar puncture showed a normal result. Afterwards an enlarged spleen was found and a diagnosis of leukemia was put forward. The patient thereupon went to Frankfurt where a low blood sugar was found during the attacks. The spells disappeared after administration of glucose, they could be induced by injection of insulin. Consequently, a diagnosis of islet cell tumor of the pancreas was established. In the same hospital an arthritis deformans of the hip joint with destruction of the head of the femur was also found for which an orthopedic support was prescribed.

As the condition became worse, the patient was operated on and a tumor the size of a nut was removed from the pancreas. On microscopic examination this appeared to be an islet-cell tumor. After this operation the attacks of hypoglycemia disappeared. An explanation for the enlarged spleen was not found, the condition of the left leg remained unchanged.\*

Family history revealed that the patient's father died of arteriosclerosis, his mother of kidney trouble. One sister has gall stones. She showed no clinical signs of Gaucher's disease on examination by one of us. One brother is suffering from Gaucher's disease. The diagnosis was established on clinical grounds and verified by sternal puncture elsewhere. A second brother is reported as suffering from a slightly enlarged spleen and a moderate secondary anemia. A third brother died when he was five months old. A cousin on the maternal side also suffered from Gaucher's disease, the diagnosis was said to have been established by pathological examination of an extirpated spleen.

*Physical examination.* The patient appeared in a moderately good state of health. The skin of the face was yellow, but there was no jaundice. Small pingueculae were present in both eyes. Skin and mucous membranes were somewhat anemic. Around both ears the skin showed some vitiligo. The skin of the entire body was somewhat pigmented, probably partly due to exposure to the sun. Examination of the eyes revealed a bilateral myopia of 2.5 diopters. Otherwise, neck, heart and lungs were all normal. The abdomen was diffusely swollen. The liver was palpable, two fingers below the costal margin, with

\* The case up until this stage has been described by Reiter.\*

rounded edge and smooth surface. The spleen was markedly enlarged, the organ reached downward to the level of the umbilicus and had a rounded edge. Both organs were firm in consistency and not tender.

The left leg was considerably shorter than the right, it was supported by an orthopedic apparatus. All movements in the left hip joint were restricted, especially adduction and rotation. Movement in the right hip was completely free. There was no tenderness on pressure. There was no abnormal pigmentation of the legs. Reflexes were completely normal.



FIG. 1, CASE 2. LOCALIZED AREAS OF RAREFICATION IN BOTH FEMORA

The urine contained some urobilin but no further abnormalities. The sedimentation rate was normal. The blood picture showed hemoglobin, 85 per cent, erythrocytes, 5,300,000, leukocytes, 4,700, blood platelets decreased. The lipid spectrum showed free cholesterol 44.5 mg per cent, cholesterol esters 99.1 mg per cent, total cholesterol 143.6 mg per cent, lipoid nitrogen 24.7 mg per cent, lipoid phosphorus 6.29 mg per cent, total lipid and fat (without cholesterol) 269.4 mg per cent.

On roentgenological examination the head of the left femur appeared flattened and partly destroyed.

In the head and in the shaft of both femora several areas of rarefaction were present (fig 1) The lower ends of both femora were swollen, (fig 2) the left tibia showed several areas of decalcification (fig 3)

Microscopic examination of the sternal marrow showed the typical Gaucher's cells

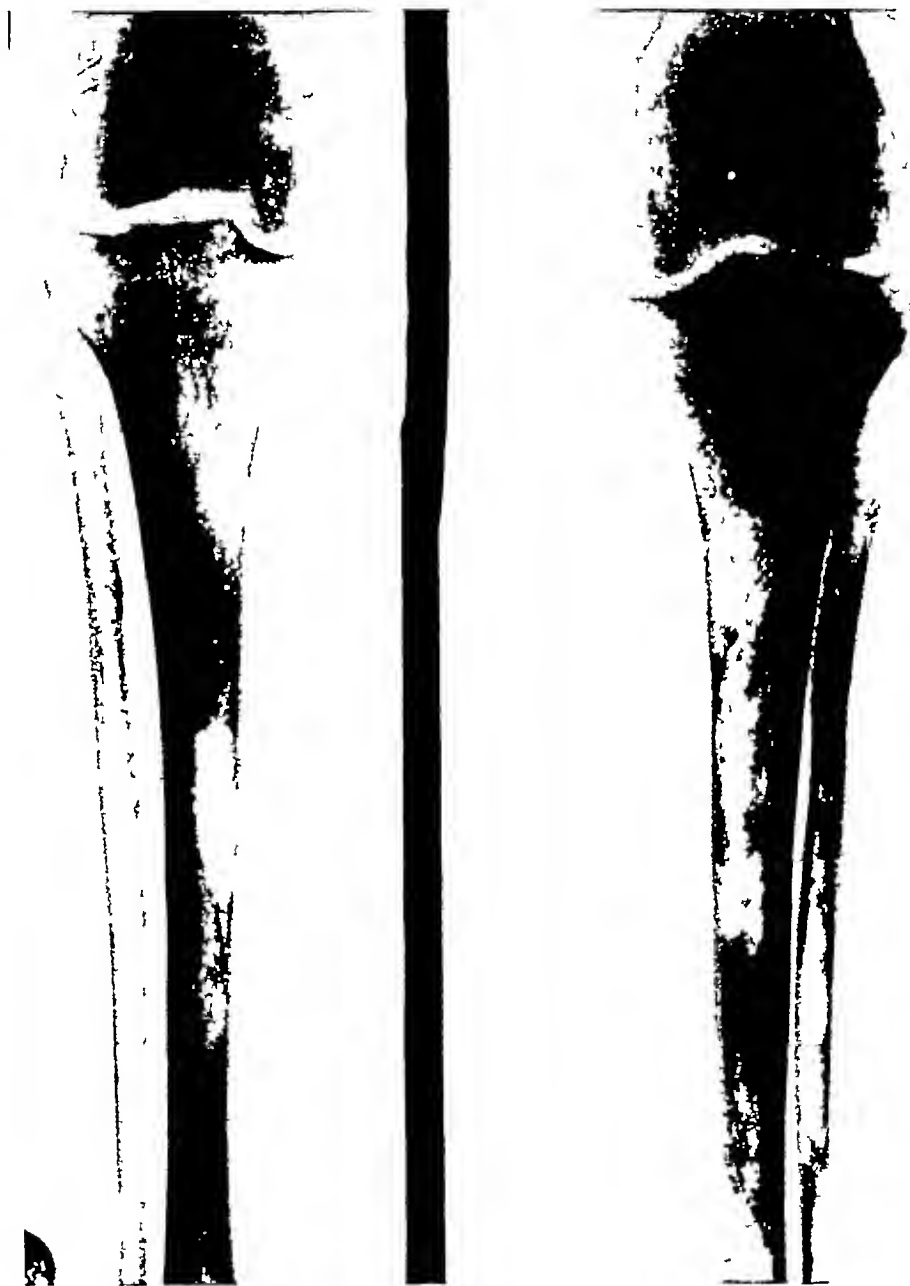


FIG 3, CASE 2 LOCALIZED DECALCIFICATION IN BOTH TIBIAE

The patient was put on a vegetarian diet. Except for occasional periods of sciatica he had been relatively free from symptoms when in 1940 he suffered a spontaneous fracture of the shaft of the right femur which brought him to the hospital again. It was then found that the bone lesions had increased.

in many areas. The fracture was treated by surgical extension and healed with satisfactory callus formation (fig. 4). However a bone abscess formed on the spot where the nail had been driven through



FIG. 3, CASE 2. LOCALIZED DECALCIFICATION IN BOTH TIBIAE

the head of the tibia. The pus contained some large pale cells that might have been Gaucher cells. The abscess healed after drainage and the patient's condition since has been stationary. Later he gave up the diet and this did not seem to make any difference in the slow course of his condition.



FIG 4, CASE 2

Spontaneous fracture healing with callus formation of the right femur. Localized decalcification and swelling of lower end of the femur (Erlenmeyer flask appearance)

## CASE 3

Mr Fl, a 30 year old Jewish business man of German birth presented himself for the first time on March 25 1938

*History* For one year he had had increasing difficulty in walking because of pain in the left knee and left buttock, which had become almost unbearable in the last few months. The patient did not present these complaints in connection with another disease from which he had been known to have suffered

for at least twenty years. Since that time he had seen several doctors, because of frequent hemorrhages from the nose, diarrhea and occasional fever. He had been told that he had a large liver and spleen. There were no known cases of a similar disease in his family. His mother was examined and found to be normal. His father had died from a heart attack. The patient was an only child. He was married to a normal wife. He had one child, a boy, five years old, who was found to be normal.

When the patient was 10 years old he was operated on for appendicitis. In 1916, he suffered from pericarditis. Tonsils and adenoids had been removed in 1926, the operation was followed by severe hemorrhage.

*Physical examination* The patient appeared to be in moderately good health. He had a myopia of both eyes (D.4). There were no pingueculae, the pupils were normal. There was no abnormal pigmentation of the face. Mouth, throat and neck were normal. The abdomen was greatly distended. The spleen protruded from under the costal margin and reached almost the inguinal ligament. The liver was also enlarged and firm and almost reached the umbilicus.

Reflexes were normal. The left leg was about 2 cm. shorter than the right. Movement in the left hip joint was extremely painful and greatly restricted. There was pain on pressure in the hip and in the groin. There was no abnormal pigmentation on the legs.

The urine contained some urobilin, but was otherwise normal. The stools contained no occult blood. The sedimentation rate was 12 mm. after one hour (Westergren). The blood picture showed hemoglobin, 64 per cent, erythrocytes, 3,400,000, and leukocytes 3,000. Only a few thrombocytes were present in the smear. Calcium, inorganic phosphate and phosphatase and nonprotein nitrogen values in the blood were normal. The lipid spectrum was essentially normal: free cholesterol, 55.6 mg. per cent, cholesterol esters, 66.6 mg. per cent, total cholesterol, 122.2 mg. per cent, lipid phosphorus 6.51, lipid nitrogen 27.82 mg. per cent, total lipid fats (without cholesterol) 276 mg. per cent. The patient was put on a low calcium diet for balance studies. No abnormalities in the excretion of calcium or phosphate in the feces were found, but the excretion of calcium in the urine was low, 44, 58 and 94 mg. per 24 hours, respectively, on three consecutive days.

X-ray examination showed destruction of the head of the left femur. Sternal puncture revealed the presence of many Gaucher cells.

*Course* The left leg was immobilized in a plaster cast. After six months the local condition seemed improved. The pain disappeared, and except for occasional attacks of sciatica the patient managed to go about his business. From 1939 onward he began to suffer from attacks of severe pain in the left side of the chest and abdomen, which were followed each time by periods of fever. On one occasion a splenic friction rub was heard. It was felt that these attacks were due to splenic infarcts with perisplenitis. The attacks recurred so often that the patient was practically confined to bed. In view of this situation, splenectomy was performed in June of 1941. The operation was extremely difficult due to extensive adhesions between the spleen and the diaphragm. Profuse bleeding occurred, and the patient succumbed 12 hours postoperatively, in spite of repeated transfusions. Permission for postmortem examination could not be obtained. The spleen showed the typical picture of Gaucher's disease. Unfortunately, the chemical investigation was interrupted when the chemist was deported to a German concentration camp from which he did not return. The dried spleen was lost on this occasion.

#### CASE 4

W. de B., born in 1909, a male office clerk, aged 25, the only patient in this series who was not Jewish, was first seen by one of us in February 1934, when he was admitted to the Binnengasthuis because of abdominal discomfort.

*History* Apart from scarlet fever, diphtheria, and measles, he had never been ill. For several years he had noticed an increasing tendency to bleed. His sister, the only living member of the family, was normal. There was no history of any other member of the family having had a similar disease.

*Physical examination* There was a slight malar flush and bilateral pingueculae. Both eyes were myopic. The chest was normal. Both the liver and spleen were enlarged, the latter reaching the anterior superior iliac spine. His legs showed a peculiar patchy brown pigmentation which ended sharply at the instep of each foot. The urine was normal. For laboratory examination (leukopenia and thrombocytopenia) the reader is referred to the first publication.\*

\* This case was reported (Case 5) by Bloem, Groen and Postma.<sup>2</sup>

Since 1934 his condition remained fairly satisfactory on a vegetarian diet, but on June 28, 1938, he was admitted to the department of medicine of this hospital because of sudden severe pain in the sacral region after slight trauma

On physical examination, it appeared that the liver and spleen had increased in size. These organs were in close contact in the left mamillary line, where they could be felt to rub against each other. A typical friction rub could be heard over the spleen. The pigmentation of the legs had also increased in area and intensity.

Liver function tests (galactose and bromsulphalein excretion) were normal. Vandenberg (indirect), 0.96 mg per cent. Sedimentation rate, 61 mm after one hour.

The blood picture showed hemoglobin, 59 per cent, erythrocytes, 3,100,000, and leukocytes 2,100.

#### *Differential*

Segments	61
Stabs	2
Monocytes	5
Lymphocytes	29
Eosinophiles	2
Basophiles	1
Thrombocytes	80,000

Toxic granulation in about 30%, marked anisocytosis, poikilocytosis and polychromasia.

In 1934, a diagnosis of Gaucher's disease had been made on clinical grounds only, this time a sternal puncture was performed. The typical cells were found.

X-ray examination showed no gross abnormality of the bones. The pain in the back disappeared rapidly after rest in bed and physical therapy.

The patient went home without pain but otherwise his condition had remained unchanged. He was a great believer in the favorable influence of a purely vegetarian diet on his condition. His diet consisted of whole wheat bread, oleomargarine, jam, vegetables, potatoes, peas, beans, nuts and fruit. He gradually lost a good deal of weight on this diet. He developed muscular atrophy and his muscular strength diminished markedly. He also developed pitting edema of the feet. The possibility that this might be due to a lack of animal food was pointed out to him but he continued on his exclusive vegetable diet. He died at his home in 1939 in a state of general exhaustion, possibly from protein deficiency.

#### CASE 5

R. P., a 43 year old Jewish single female school teacher was admitted in 1938 for recurrent attacks of sciatica. These attacks had started five years ago in the right leg, lasting only for about a fortnight. The pain radiated from the buttock along the back of the leg to the lateral side of the lower leg. Later she had similar periods of pain in the left leg. Gradually the pain became more severe, it lasted a longer time and the patient could hardly walk.

Apart from this she had very few complaints. She had noticed that her abdomen was a little swollen but she had attributed this to her getting fat. She had also noted a tendency to bleed, which was especially cumbersome during menstruation.

*Physical examination* revealed myopia, malar flushes, a somewhat increased second pulmonic sound, enlarged spleen which reached down as far as the umbilicus, a liver which was about 1 or 2 fingerbreadths enlarged, and a diffuse yellowish tinge of the skin of the whole body. There was a typical Lassegue sign bilaterally. Reflexes and sensitivity were normal. Movements such as turning around in bed, were extremely difficult. The blood picture showed leukopenia and thrombopenia, with little or no anemia. Numerous Gaucher's cells were found in the sternal marrow.

*Family history* did not reveal any known cases of Gaucher's disease. The patient's mother had died of carcinoma of the uterus. The father was examined and found to be normal. She was one of twelve siblings. Two of the siblings who could not be examined had a suspicious history of Gaucher's disease. The disease however was found with certainty in a younger brother (case 6).

*Course* The patient's condition fluctuated. When one day she developed a typical biliary colic with jaundice. She was operated upon and a gallbladder full of stones was removed. She made a satisfactory recovery but her sciatica kept on troubling her from time to time. In 1943 she was arrested by the Germans and transferred to a concentration camp from which she returned with a pulmonary tuberculosis.



## CASE 6

M P, a brother of Case 5, a 42-year old married Jewish business man, had no complaints. One year ago he had had an attack of sciatica which lasted for only ten days.

On physical examination, the spleen was found to be two fingerbreadths below the costal margin and the liver was one fingerbreadth enlarged. He had myopia and malar flushes exactly similar to those of his sister. These flushes were not presented in the nonaffected members of the family. Sternal puncture revealed Gaucher cells. His wife was examined and found to be normal. Two children, 6 and 3 years old, respectively, were apparently normal.

The patient's condition was followed. He had no complaints when he was seized by the Germans and deported to Poland. He did not return.

## CASE 7

A X, a Jewish girl, born September 15, 1923, was seen for the first time in January, 1939, through the courtesy of Professor Van Creveld.\*

*History* When she was two years old, a swelling of the abdomen was noticed. Professor de Lange, who was the first to make the diagnosis in 1929, found an enlarged spleen and liver, hypochromic anemia, leukopenia and thrombopenia, with marked hemorrhagic diathesis. There were no changes in the bones at that time. Gradually the typical Gaucher pigmentation developed on both legs.

The diagnosis of Gaucher's disease could be made with certainty because in a sister (E X, Case 8) splenectomy had been performed and Gaucher cells had been found in the spleen.

Since the report in 1931 by de Lange,<sup>13</sup> the patient's illness progressed steadily<sup>14</sup> so that when we saw her the liver and spleen had become excessively enlarged. There were also radiologic signs of involvement of the bones. In the sternal marrow many typical Gaucher cells were found.

*Course* The condition had become gradually worse when the patient and her parents were deported in 1942 to a German concentration camp from which they never returned.

## CASE 8

E X, a Jewish girl, born March 19, 1927. She is a sister of the patient reported above (Case 7). She was also described in de Lange's papers. She was first seen in April, 1939, through the courtesy of Professor van Creveld.

*History* This child showed an enlargement of the spleen at an early age. Splenectomy was performed in 1930 and Gaucher cells were identified in the spleen. A detailed chemical examination of the spleen was carried out by Westenbrink<sup>35</sup> who isolated and identified kersin.

In 1938, she began to complain of pain in the left leg, and fever. Extensive cystlike rarefactions were present in the x-ray pictures of the left femur. Similar attacks of pain recurred regularly.

*Physical examination* The patient showed slight generalized swelling of the lymph glands and a very large liver of firm consistency, reaching down to the umbilicus. Both lower legs showed the typical leaden brownish-grey pigmentation. Liver function tests were normal. It is curious to note that in this case (as in Case 1 where splenectomy had also been performed) the typical leukopenia was replaced by a slightly high leukocyte count. The blood picture was as follows: hemoglobin, 80 per cent, erythrocytes, 4,570,000, leukocytes, 10,400, platelets 120,000.

*Differential*

Segments	31 5
Stabs	3 5
Monocytes	5
Lymphocytes	60 0
Eosinophiles	0
Basophiles	0

The blood chemistry findings were essentially normal. Sternal puncture revealed the presence of many typical Gaucher cells.

*Course* This girl shared the fate of her sister.

\* Cases 7 and 8 have been described by de Lange<sup>13-15</sup> and Heilbron.<sup>7</sup>

## CASE 9

The occurrence of Gaucher's disease in two sisters (Cases 7 and 8) naturally focused our attention to the possible presence of the disease in one of the parents. Both had been examined in 1931 by de Lange and on that occasion had not shown any physical abnormality.

On re-examination, the father (P X) had no complaints at all. In addition to a chronic bronchitis, we found pingueculae on both eyes and a few scattered grey spots on the skin of the legs.

The mother (E X-C) gave a history of prolonged bleeding after slight injury and during menstruation. No abnormalities were found on physical examination.

Hematologic examination in both cases yielded normal results.

Sternal puncture was carried out in both parents. No typical Gaucher cells were found but the father's marrow contained a rather high percentage of unclassified cells of the type most authors would call reticulum cells or stem cells. Some of these resembled in appearance Gaucher cells but they were smaller in size. The nuclei of these cells were rather small and in some cells they were shifted to the periphery. The protoplasm stained faintly and was inhomogenous in character. It contained a few irregular vacuoles. Occasionally the cytoplasm resembled in its structure compressed tissue paper. Because of the presence of these cells it was felt that the father of cases 7 and 8, who did not show the clinical characteristics of Gaucher's disease, actually suffered from that disease in a minor degree. Apparently it was he who had transmitted the disease to two of his children where what had been a latent abnormality in his case had given rise to a severe clinical picture at an early age in theirs.

The family tree of Cases 7, 8 and 9 could not be completed. The two girls had three brothers. All were examined clinically and by sternal puncture. In no way was any evidence of Gaucher's disease obtained in them. Other members of the family on either the father's or the mother's side could not be examined. So far as could be ascertained by questioning, there was no case with the clinical features of Gaucher's disease among them.

## CLINICAL SYMPTOMATOLOGY

The cases here presented, together with those earlier described by one of us<sup>2</sup> illustrate most of the salient features of the disease. Many clinicians who have not had a wide experience with this rare malady think of Gaucher's disease as a more or less fixed clinical picture. The reverse is true. There is a remarkable variability in many respects. The disease may make its onset early in infancy, in childhood or early or late in adult life. Its progress may be slow or rapid but it is important to know that its course is always progressive. 'Spontaneous remissions' occur during times when the patient may be relatively or entirely symptom-free but the objective findings have never been observed to regress. As a matter of fact it is the rule that patients do not develop any complaints at all until relatively late, when the spleen and liver have already increased to a considerable size. During the 'silent' or 'subclinical' stage the subject is unaware of his condition until a medical examination happens to take place. In some instances of the present series the disease was discovered only when the relatives of a known case were examined.

Although the disease process seems to be going on continually, the rate of progression varies considerably from patient to patient. The earlier the onset, the more rapid seems to be its course. Cases observed early in infancy almost invariably die within the first year<sup>15</sup>, persons with Gaucher's disease in childhood seldom grow older than 50 years, when the disease becomes manifest late in life it does not seem to shorten the lifespan of the individual.

The localization may also show considerable variation. Although spleen and liver are the most prominently involved it is probably safe to say that Gaucher's disease hardly ever spares the bone marrow. However, the presence of the Gaucher

cells does not necessarily upset the *macroscopic* structure of the bone, and thereby the disease may escape detection by radiologic examination, even when bone-marrow puncture or postmortem examination<sup>7</sup> proves that the cells are there. In the later stages, when massive aggregations of the cells occur, the bones usually show generalized or local osteoporosis.<sup>10</sup> Even in the advanced cases, however, the degree of macroscopic affection of the bone is variable and through this we may encounter considerable variations in the clinical picture. Repeated attacks of low back ache, radiating along the sciatic nerve, either unilaterally or bilaterally, may be the first complaint (cases 5 and 6). Or patients suffering these "osseous forms" may enter the hospital with a spontaneous fracture (case 2) or for what seems a purely orthopedic problem like "arthrosis" of the head of the femur (case 3). Bone abscesses occurred in case 2 and 7.<sup>14</sup>

Next to localizations in spleen, liver and bones, foci of Gaucher cells have been found in the lungs,<sup>19</sup> the kidney,<sup>9</sup> and the brain,<sup>29</sup> but these sites are rare.

Complications are frequent in this disease and they also depend on the varied localization. Pigmentation of the legs has been referred to<sup>2</sup>, it occurs relatively late in the disease and has been observed to disappear.<sup>14</sup> Cachexia supervenes in the long standing cases. Case 1 died under a clinical picture of cor pulmonale, possibly due to accumulation of Gaucher cells in the lungs. Other cases we have seen had an increased second pulmonary sound, as a possible indication of pulmonary hypertension. Most remarkable was the combination of Gaucher's disease with an islet cell tumor of the pancreas in case 2. The clinical picture in case 3 was dominated by the successive episodes of splenic infarcts which gave rise to perisplenitis and adhesions around the spleen. Bleeding from these adhesions after splenectomy caused the death of this patient. Case 5 suffered mainly from "symptomatic sciatica". She also had gallstones, a combination we have seen in other cases. She later developed tuberculosis as did Gaucher's original case.<sup>6</sup> In two cases of this series a splenectomy was carried out successfully. The impression was that the operation does not significantly alter the course of the disease. It certainly did not stop its progress. Interestingly enough, these patients' leukopenia changed into a slight but permanent leukocytosis, whereas the thrombocytopenia and hemorrhagic diathesis remained. Our experience confirms the opinion of several authors<sup>14</sup> that splenectomy should be carried out only if the spleen itself gives rise to local symptoms which justify its removal. If a patient, as was instanced with case 3, has had repeated attacks of perisplenitis, the surgeon should be prepared to find extensive adhesions between the spleen and the diaphragm. This situation combined with the hemorrhagic tendency caused the death of case 3. Treatment with a purely vegetarian diet appeared to be just as unsatisfactory as splenectomy. Several of our cases have been treated with this diet. The assumption was that by cutting down the exogenous supply of lipoids in the diet, a slowing down in possible storage of the Gaucher substance could be induced. The results have not borne out any support for this hypothesis and it is not unlikely that the restricted diet has hastened the death in case 4.

## THE STERNAL MARROW IN GAUCHER'S DISEASE (FIG 7)

In all our cases, the sternal puncture was carried out in the manubrium. Films were made and stained with the May-Grunwald-Giemsa stain. We recommend that several *thin* films be made. The cells should be looked for especially at the end of the smear. The best search for the presence of Gaucher cells is made with a low power lens without oil immersion (fig 5). The typical cells will catch the eye at once by their unusual size, usually 30 or 40 $\mu$ , although there may be considerable variation in size. The cells possess one or two nuclei that are usually situated eccentrically. In the smaller Gaucher cells, the nucleus is sometimes in

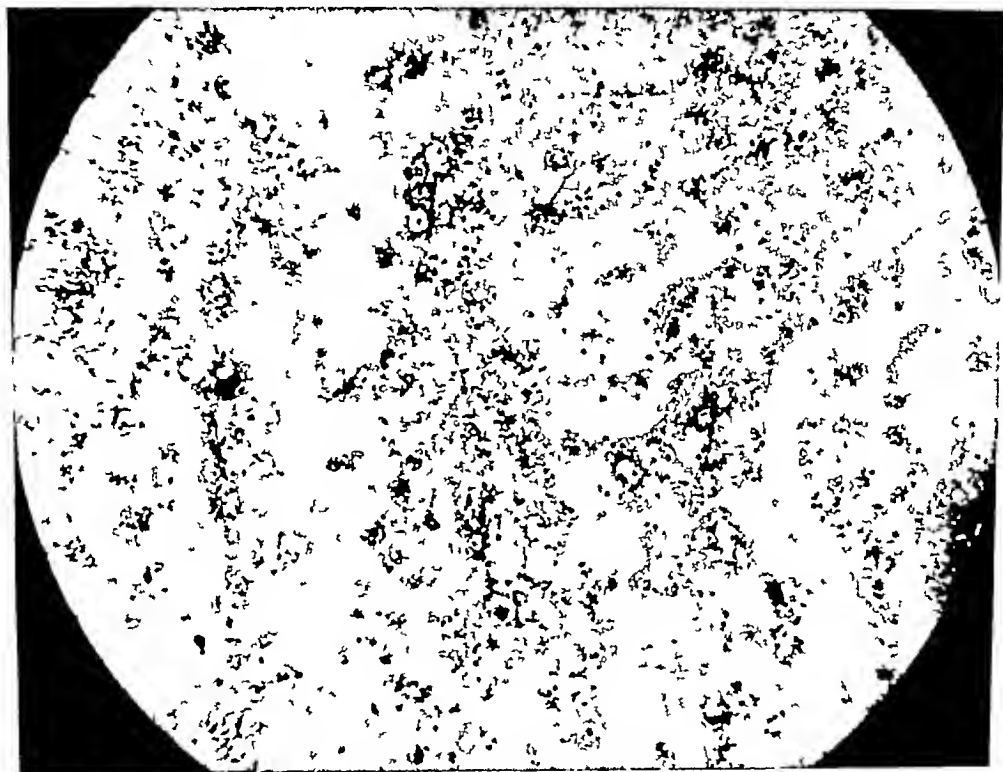


FIG 5, CASE 3. The aspect of the sternal marrow smear under low power. The Gaucher cells catch the eye by their large size.

the center, in the larger ones it is always displaced toward the periphery. The nuclei are relatively small with a well-stained nuclear membrane. They often contain a nucleolus. The nuclei of the Gaucher cells resemble the nuclei of those cells in the bone marrow which are referred to by some authors as reticulum cells, by others as stem cells, and which are found in a small number in normal bone marrow. As a matter of fact, it is not unusual to find an increased number of these reticulum cells in the marrow smear of patients with Gaucher's disease. Even more commonly one finds cells that are somewhat intermediary in type between the reticulum cell and the Gaucher cell. They have the size of the reticulum cell, or are a little larger, the protoplasm has the faint staining of the reticulum cell but it is more meshy in structure. If they were seen in normal bone marrow one would

call them reticulum cells, in the presence of typical Gaucher cells, one is inclined to regard them as small atypical Gaucher cells. They are probably reticulum cells that have taken up less kersasin and therefore have not changed in type as completely as the typical Gaucher cell. They be might called 'early' or 'young' Gaucher cells. This view is in accordance with the opinion of most pathologists,



FIG. 6, CASE 3. Gaucher cells under high power illustrating the wrinkled tissue paper appearance of the protoplasm. Note that there is a marked difference in size amongst the Gaucher cells.

who have found similar transitory types between Gaucher cells and reticulum cells in the spleen and therefore regard the Gaucher cells as a transformed reticulum cell (Mandlebaum and Downey<sup>18</sup>).

The cytoplasm of the Gaucher cell occupies by far the larger part of the cell body, it stains slightly gray or bluish. In the typical cells it shows a coarse

mesh as if it consisted of compressed tissue paper (fig 6) Sometimes the protoplasm contains vacuoles so that it resembles foam cells <sup>Erf<sup>6</sup></sup> by the use of the supravital staining technic has shown that these vacuoles are artefacts produced during the fixation process The cytoplasm of the Gaucher cell does not stain with any

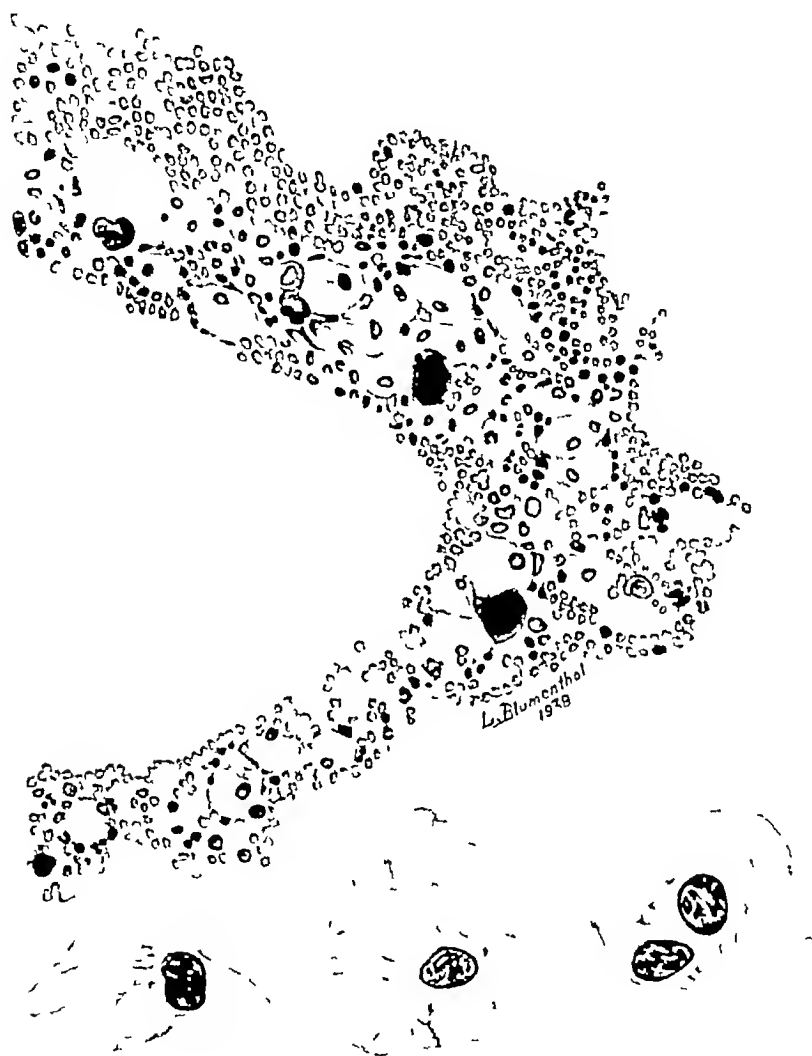


FIG 7, CASE 3 A drawing of the sternal marrow in Gaucher's disease Three Gaucher cells are drawn separately under high power

of the known dyes, neither does it take the sudan or Goldman's lipid stain or oxydase stains The protoplasm does not show double refraction All these properties fit in with what we know about the characteristics of the lipid kersin that has been isolated from organs containing Gaucher cells

Many authors have reported the finding of Gaucher cells in the bone marrow of living cases Some have used biopsies of the tibia,<sup>34 1 25 16</sup> but the majority have preferred to use sternal puncture<sup>28 33 32 5 22 31 21 8 36</sup> Only in a very few cases has the puncture failed to reveal the presence of the typical cells Indeed, one might

very well call the sternal puncture the only method that will enable the clinician to establish a positive diagnosis of Gaucher's disease with certainty. The cells distinguish themselves from all normal bone marrow cells with great ease by their size and structure. The only difficulty that might present itself is in differentiating the cells from the large cells that occur in Niemann-Pick's disease, but the protoplasm in the Gaucher cells is characterized by a fibrillar, meshwork, whereas the Niemann-Pick cells have a foamy appearance.

It is significant that one also finds the Gaucher cells in the marrow in those cases where no gross lesions seem to be present in the bones on x-ray examination. This proves that in most cases the cells are almost universally scattered throughout the bone marrow. It is only in those places where the cells accumulate closely enough to produce atrophy of the bony trabeculae and thinning of the cortex that a "nidus" of some magnitude is formed. It follows that the distinction between visceral types and osseous types of the disease<sup>24</sup> has only a relative, and no absolute, meaning. In fact, cases 7 and 8 were described first as purely visceral in type while marked bony lesions developed later. This happened in two sisters, while splenectomy had been performed in only one of them. Splenectomy apparently was useless as a measure against the progression of the disease in the bones.<sup>34 14</sup>

The importance of sternal puncture as a diagnostic procedure in Gaucher's disease manifested itself especially in the elucidation of the hereditary mechanism in cases 7, 8 and 9. Neither father nor mother of the girls showed clinical evidence of the disease, yet the fact that two of their children were affected could only be interpreted by the assumption that one of the parents was a 'carrier'. By the detection of atypical reticulum cells, resembling Gaucher cells in the father's bone marrow, this assumption was substantiated. This, then, appears to be one of the most important advantages of the sternal puncture, viz., the possibility of establishing an early diagnosis in individuals where the clinical picture is not fully developed. In this connection, the cases of Vogel, Erf and Rosenthal,<sup>28</sup> Petit and Schleicher,<sup>23</sup> and Morgans<sup>20</sup> are especially interesting. These patients showed no enlargement of spleen and liver (as yet) but only involvement of the bones. Without sternal puncture the diagnosis would have been impossible.

Emile-Weill, Isch-Wall and Perles<sup>33</sup> have advocated splenic and even hepatic punctures as diagnostic procedures. As a matter of fact, hepatic puncture was used by one of us<sup>2</sup> on a former occasion. However, we feel that the sternal puncture has all the advantages of ease and safety and none of the drawbacks associated with the splenic or hepatic puncture in these patients in whom we know that there is a tendency to bleed.

### SUMMARY

The authors present nine cases of Gaucher's disease in which the diagnosis, suspected on clinical grounds, was definitely established by the detection of the Gaucher cells in the smear of the sternal marrow. The authors review the varieties of the clinical picture in this disease. They discuss the importance of sternal puncture with special reference to the possibility of establishing the diagnosis in subclinical cases.

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# THE HEREDITARY MECHANISM OF GAUCHER'S DISEASE

By J GROEN, M D

THE NUMBER of cases of Gaucher's disease that have been described up to the present time is about 250, but it is probable that many more cases have been observed. Collier's case<sup>7</sup> was probably the first in which more than one member of the family was found to be affected. After him, Bovaird,<sup>4</sup> in 1900, described the disease in two sisters. Since then, the familial occurrence of Gaucher's disease has been noted by several observers. About one-third of the published cases have occurred in members of the same family so that a hereditary factor is undoubtedly present. However, the family trees which have been published are quite few, and the exact mechanism by which the disease is inherited is still unknown.

The present author has observed 21 cases of Gaucher's disease. Seventeen were familial and occurred in six different families. Four more cases in these families could not be examined but the available evidence was sufficient to accept them as cases of Gaucher's disease, which brings the total number of familial cases discussed in this report to 25. Four other patients were seen in whom no other members of the family were affected. These were considered to be so-called 'sporadic' cases. The clinical features of our patients have been described elsewhere.<sup>3, 9</sup> It is the purpose of this paper to review the scanty data on the heredity of Gaucher's disease from the literature and to present the family trees of the cases which have been observed by the author. In reviewing the literature excellent summaries have been found in the papers by Brill, Mandelbaum and Libman,<sup>5</sup> Hoffmann and Makler,<sup>11</sup> Rowland,<sup>16, 17</sup> Reiss and Kato,<sup>14</sup> De Lange,<sup>12</sup> Atkinson<sup>2</sup> and Thannhauser.<sup>18</sup> Atkinson's admirable review of the cases of Gaucher's disease described in children appeared in 1938 and has been especially valuable.

In considering the familial cases in the literature, one is impressed by the following facts:

1. There have been 64 familial cases reported which occurred in twenty-five families. The inclusion of the cases described in this report would bring the total up to 89 in thirty-one families.

2. In twenty-three of the families in the literature, the disease occurred in a 'horizontal' spread only. By this is meant that the disease appeared to be present in the members of one generation only, namely in brothers and sisters or in cousins, but not in parents or grandparents or in the offspring of the patients. This peculiarity in the familial incidence of Gaucher's disease is underlined in a statement by Hoffmann and Makler that in no series of children has either of the parents suffered from the disease nor has it been transmitted from any adult to a child.

3. In only two families was there a 'vertical' occurrence described.<sup>6, 16</sup> In these families the disease was found to be present in two generations, namely, in Rettig's case, in a father and his daughter,<sup>15</sup> and in the case described by Bychowski, in a father and three of his children.<sup>6</sup> Quite recently Morgans<sup>17a</sup> published a third

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family in which there was suggestive evidence of a vertical spread of Gaucher's disease

4 In these instances of a 'vertical' transmission there was a direct continuity of the disease from one generation to the other. Only one example of "skipping" of a generation has been described in a paper by Anderson.<sup>1</sup> This case is often quoted but the evidence offered does not seem convincing. Anderson observed four sisters with Gaucher's disease. The father and mother of these patients were found to be normal. The grandparents were not examined but it was reported that the paternal grandmother and two of her sisters had large abdomens and a yellowish brown discoloration of the skin and died of a common cause. The author did not go beyond suggesting the possibility that the grandmother and her sister might have had the disease. It would seem that this evidence is too scanty to warrant the far-reaching conclusions that have been drawn from it by some authors.\*<sup>3, 10</sup>

5 The disease does not show a preference for either sex. Atkinson found among 108 cases in children, 50 males and 53 females, the sex of the other 5 cases was not given. Of the author's present series of 25 cases 14 were males and 11 females.

6 Marriage between relatives had not taken place in the ancestry of any of the reported cases of Gaucher's disease.

7 In the families in which Gaucher's disease occurred, there have been a number of normal siblings as well as affected cases.

8 Little is known of the offspring of patients with Gaucher's disease. Many of the cases die before they reach the reproductive age, others do not marry or if married have no children. Furthermore the incidence of abortions and of stillborn babies among the offspring of the reported cases of Gaucher's disease seems to be unusually high.

The survey of the author's cases confirms many of the points mentioned above. In addition it contains some data that may help to answer some of the questions that remained open so far.

## DESCRIPTION OF FAMILIES OBSERVED BY THE AUTHOR

### FAMILY A

H. U., a Jewish merchant, aged 49, was admitted in February, 1934. He had a typical clinical picture of Gaucher's disease and the diagnosis was established by pathologic examination of a liver biopsy. The patient's father committed suicide. No further information about him was available. His mother had died of old age. The family doctor assured us that he had never found a large liver or spleen in her. Two brothers and two sisters were examined and found to be normal. His wife was normal. She had had no abortions. They had two children, both died shortly after birth.

He is an example of a sporadic case, the only one of five siblings, originating from what appeared to be normal parents, who had Gaucher's disease. The fact that both his children died shortly after birth is probably significant.

### FAMILY B (FIG. 1)

No medical information was available concerning the grandfather (I<sub>2</sub>) of this family. He had been married twice. His first wife was reported to have died of apoplexy. Nothing was known about his second wife.

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\* In the paper by Bloem, Groen and Postma,<sup>3</sup> it was said that Anderson's family suggested that the inheritance of Gaucher's disease might be of recessive character. Bloem and Postma now agree with the author that this statement should not have been made.

He had three children by his first marriage (II 1-3)

II 1 Female, unmarried, died in 1932, aged 75, of degenerative heart disease. She had been repeatedly hospitalized and no signs of Gaucher's disease were found on any occasion.

II 3 Female, still alive, 75 years of age when we examined her. She was found to suffer from gall stones and chronic cystitis but there was no evidence of Gaucher's disease. Her daughter (III 8) and two grand-daughters (IV 14-15) were examined and found to be normal.

II 2 Male, died in 1922, aged 62, of pulmonary embolism, after prostatectomy. He had once been told by a doctor that he had a big liver but the surgeon who operated on him had found nothing abnormal on an (admittedly superficial) examination of the abdomen. He had had no abnormal tendency to bleed. His wife had a mild diabetes but otherwise she was found to be normal. They had eight children (only seven of whom are represented in figure 1).

III 1 Female, 51 years old, was found to have a chronic cystitis but was otherwise normal, as were her three children (IV 1-3).

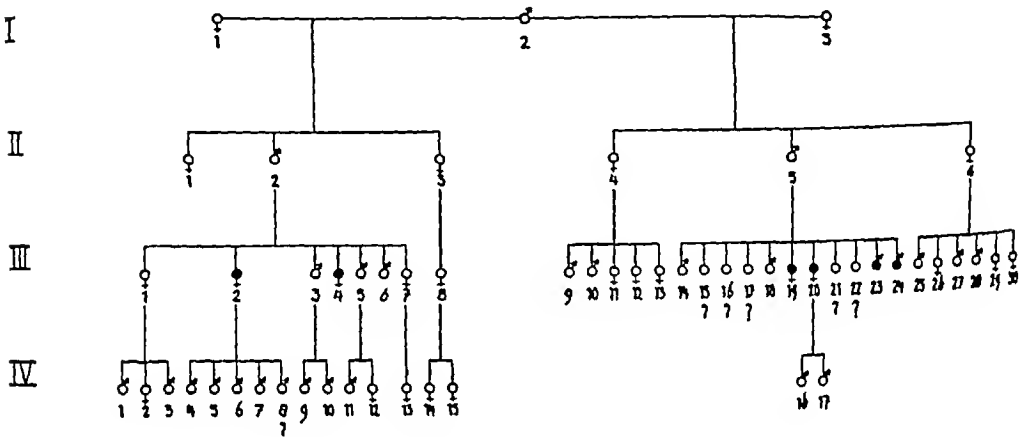


FIG. 1. FAMILY B

III 2 Female, was known to her doctor to have a very large spleen and leukopenia. She suffered from nosebleeds. She died at the age of 36 from a severe postpartum hemorrhage on giving birth to her fifth child. This fifth child died of unknown cause one day after birth. The other four children were found to be normal. We feel that she very likely had Gaucher's disease.

III 3 Male, had frequent nosebleeds and hemorrhages after tooth extraction. No clinical signs of Gaucher's disease were found. Both his sons were normal.

III 4 Female, a typical case of Gaucher's disease, with a very large spleen and liver, symmetrical pigmentation of the legs, bone lesions and typical blood findings. She is unmarried.

III 5 Male, was found to have a slight glycosuria. He was otherwise normal, as were his two children.

III 6 Male, was found to be normal. He was married but had no children.

III 7 Male, married, was found to be normal, as was his only child, a daughter.

Not included in figure 1 is one other female, who was found to be normal, as was her only child, a son.

From the second marriage of I 2, also sprang three children.

II 4 Female, had five children. None of these could be personally examined but information obtained from family doctors and from hospital records revealed no evidence of Gaucher's disease in any of them.

II 5 Male, died suddenly at the age of 50, probably of coronary occlusion. Up till then he had always been a healthy man. His wife had had hypertension, she died of apoplexy. They had eleven children (III 14-24).

III 14 A schizophrenic who always refused medical examination. He died of suicide.

III 15, 16, 17 Died during the first days of life.

III 18 Male, unmarried, was found to be normal.

III 19 Female, found to have a very enlarged spleen and liver, this was confirmed by the surgeon when

she was operated on for gall stones. She had a tendency to bleed. In 1937 she died of subdural hematoma after an insignificant trauma of the head. Although we were not able to examine her personally, she very probably had Gaucher's disease. She was married but had no children.

*III 20* Female, in 1936 she was reported by Bloem, Groen and Postma<sup>3</sup> as having gall stones but being otherwise normal. Since then the patient has withdrawn her former refusal to be examined. The present author has had an opportunity to examine her. She had a definitely enlarged spleen but refused laboratory examinations. She suffers from nose bleeds. She has two children (*IV 16-17*). It was not possible to examine them but they are reported as being normal.

*III 21-22* Were either abortions or dead children. This could not be ascertained with certainty.

*III 23* Male, was found to have an enlarged spleen and liver, myopia and malar flushes, leukopenia and thrombopenia. He refused sternal puncture. We regard him as very likely a case of Gaucher's disease. He was unmarried.

*III 24* Male, died at the age of 13, after splenectomy. The diagnosis of Gaucher's disease was made by pathologic examination of the spleen after operation.

*III 6* Female, was normal, as were her six children (*III 25-30*). None of them had attained the marrying age.

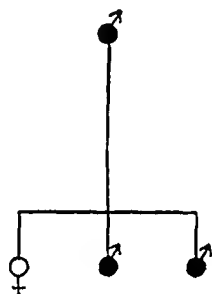


FIG. 2. FAMILY C

The interesting points in this family tree are

1. Gaucher's disease appeared among the cousins of two families who were united only by one grandfather who had been married twice. Yet this man, who was the only link between the affected families, was apparently not a sufferer, nor were his two sons, who were half-brothers. However, these men (*II 2* and *II 5*) produced two and four children, respectively, who had the disease in a marked degree.

2. One of the sufferers who married (*III 2*) had one child that died shortly after birth. Furthermore, no less than 5 cases of early infantile death or stillbirth occurred in the offspring of *II 5*, although he was not a clinical case but only a transmitter.

3. A high proportion of the patients in this family were unmarried, or they were married but had no children. As a result, no cases of Gaucher's disease occurred in the fourth generation.

4. The normal members of the third generation all had normal children (if they had any). The few Gaucher patients who produced offspring had one stillborn baby and also some normal children.

#### FAMILY C (FIG. 2)

*M S* male, a 65 year old Jewish merchant, was admitted in November of 1934. Twenty years previously he had had a severe hemorrhage after an operation for hernia and subsequently had bleeding of a

marked degree with every wound or dental extraction. At the time of this operation no abnormalities were found on physical examination, but he now had a large spleen and liver, pingueculae, myopia, malar flushes, pigmentation and a typical blood picture of Gaucher's disease. Nothing was known about his father or mother. One brother died in an accident. Two others, aged 79 and 69, were examined and found to be normal. The patient had one daughter, who refused examination, and two sons. The elder son, aged 30, was found to have bronchitis, pigmentation, malar flushes, myopia, pingueculae, and an enlarged spleen. The younger son, aged 27, had the same signs but showed in addition early pigmentation of the legs and a typical blood picture. The older son was married but had no children. The younger son was unmarried. Neither of them had any complaints at the time of examination.

This family is remarkable because the diagnosis could be established in the father and two sons. The disease manifested itself in the father late in life. At the age of 45 nothing abnormal was found in this man. In his sons, however, the clinical picture was already fully developed at the age of 30 and 27 years, respectively.

#### FAMILY D

A 40-year old Jewish housewife, who was known for many years to have a swollen abdomen and pigmentation of the face with malar flush. On examination she was found to be a typical case of Gaucher's disease. Her father and mother, aged 62 and 60, respectively, were normal on physical examination. She had one sister who was also normal. She was married but she had no children. This patient was regarded as a so-called sporadic case of Gaucher's disease.

#### FAMILY E

Male, aged 25, non-Jewish, was admitted in 1934 with a typical picture of Gaucher's disease. Later, he was readmitted and the diagnosis was established by sternal puncture. Nothing was known about his parents, who were both dead. His sister, the only other living member of the family, was found to be normal. He married but he never had any children. He was another example of a sporadic case that produced no offspring.\*

#### FAMILY F

Mrs. E. R. B., a Jewish married woman, born in 1890, had had her spleen removed in 1922. A diagnosis of Gaucher's disease was made on the specimen. She came under our observation in 1938 with a picture of cor pulmonale. The liver was enlarged. Gaucher's cells were found in the bone marrow. The patient's father had died of carcinoma of the rectum, her mother of apoplexy. Both had died in a hospital. No enlargement of the spleen or liver had been found in either of them. The patient had two sisters and one brother, who were all found to be normal. Her husband was also normal as were her two children, 25 and 19 years old, respectively. She had had no abortions. She was considered to be a sporadic case of Gaucher's disease.

#### FAMILY G (FIG. 3)

Mr. Fa., a Jewish lawyer, born in 1901, was examined in 1937. He was found to have an enlarged spleen and liver, pigmentation and areas of destruction in the bones. Gaucher's cells were found in the bone marrow. Curiously enough, this patient had also harbored another rare disease, namely, an islet cell tumor of the pancreas. The patient's father had died of arteriosclerosis, his mother of kidney trouble. One sister has gall stones. She showed no evidence of Gaucher's disease on clinical examination. One brother is suffering from Gaucher's disease. The diagnosis was established on clinical grounds and verified by sternal puncture elsewhere. A second brother is reported as suffering from a slightly enlarged spleen and a moderate secondary anemia. A third brother died when 5 months old. The patient was unmarried.

This patient further told us that a cousin on the maternal side had had his spleen removed, a diagnosis of Gaucher's disease had been established by pathologic examination of the specimen. We have not been able to verify this statement.

\* The cases described so far are the same as those in the publication by Bloem, Groen and Postma.<sup>3</sup>

## FAMILY H

A 30 year old Jewish business man, born in 1908, was examined in 1938. He was a typical case of Gaucher's disease, with enlargement of the liver and spleen, hemorrhagic diathesis, and involvement of the bones. Gaucher's cells were found in the sternal marrow. His mother was examined and found to be normal. His father had died from a heart attack. He was the only child. He was married to a normal wife. He had one child, a boy, 5 years old, who was found to be normal. This man was regarded as a sporadic case of Gaucher's disease.

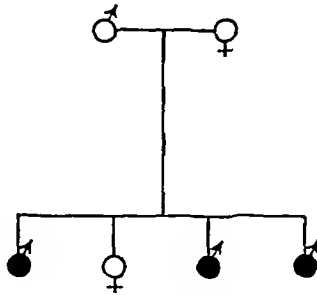


FIG 3 FAMILY G

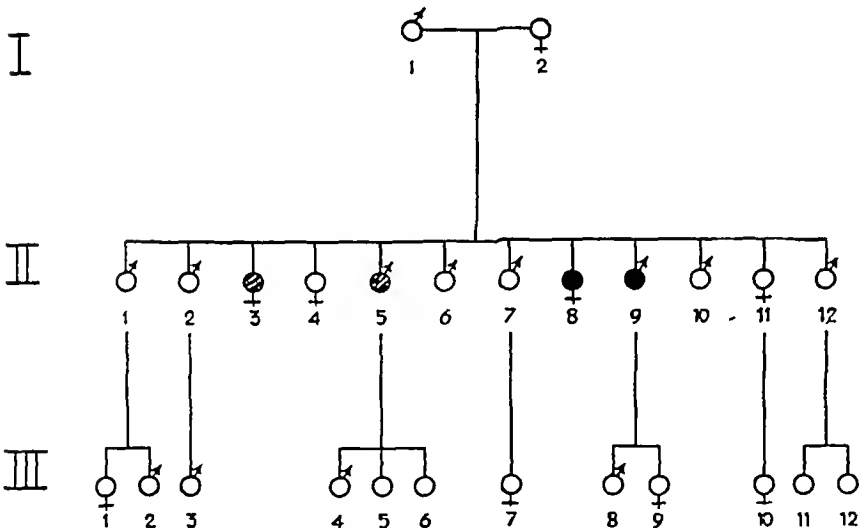


FIG 4 FAMILY I

## FAMILY I (FIG 4)

*I 1* A retired Jewish butcher who died aged 82, had a chronic bronchitis and inguinal hernia. No signs of Gaucher's disease were found in him during his life. His wife had died of carcinoma of the uterus. An internist who had examined her several times told us that he had never found a large spleen or any other abnormalities which could have indicated the presence of Gaucher's disease. They had 12 children.

*II 1* Male 55 years old, was found to be normal, as were his two children 19 and 17 years old, respectively.

*II 2* Male 8 years old died of carcinoma of the colon. No evidence of Gaucher's disease had been observed in this man by the surgeon who operated on him. His only child, a son, was examined and found to be normal.

*II 3* Female died in 1927 28 years old, after an operation for carcinoma of the ovary. No evidence of Gaucher's disease was found but the family had noticed that she had the same malar flushes as the sib-

lings II 5, II 8 and II 9. She was myopic. She died on the day of operation, possibly of an internal hemorrhage. No further evidence could be obtained and the presence of Gaucher's disease in her case must be regarded as suspicious. She was married but had no children.

II 4. Female, unmarried, was not on speaking terms with any other member of the family. She could not be traced for examination.

II 5. Male. He emigrated from Holland to the U.S.A. at an early age. He could not be examined. He was said to suffer from nose bleeds, and he had the same peculiar malar flushes as the affected siblings. His wife was apparently normal. Their first baby was stillborn. After this his wife had two abortions. We cannot go beyond suspecting Gaucher's disease in this case.

II 6. A boy, died at the age of 2 years.

II 7. Male, was examined and found to be normal except for an ulcer of the duodenum. His only child, a daughter, 7 years old, was normal.

II 8. Female, was first seen by us in 1938. She complained of severe sciatica. She was found to have an enlarged spleen and liver, and lesions in the lumbar vertebrae, the sacral and the iliac bones. She had malar flushes, myopia and a tendency to bleed. Blood picture showed leukopenia, hypochromic anemia and thrombopenia. Typical Gaucher's cells were found in the sternal marrow. In addition, she was found to have gall stones. Later she developed pulmonary tuberculosis. She was unmarried.

II 9. Male, who had never been examined previously. On examination he was found to have pingueculae, pigmentation of the skin, malar flushes, myopia and an enlarged liver and spleen. One year before examination he had suffered from sciatica but this had disappeared without treatment. Gaucher's cells were found in the sternal marrow. His two children (III 8-9) were found to be normal.

II 10. A boy, died at the age of 4 months.

II 11. Female, was found to be normal, as was her only child.

II 12. Male, had vague abdominal complaints, no clinical signs of Gaucher's disease were found. His wife had had two abortions. There were no children.

This family presented 'horizontal spread'. In the second generation there were two proven cases of Gaucher's disease and two individuals who could not be examined but in whom there was a justified suspicion of the disease. Neither of the parents of these cases had shown clinical evidence of Gaucher's disease. All but one of the normal members of the second generation, who had produced children, had a normal offspring. This one exception was an apparently normal man (II 12) whose wife had two abortions. One of the cases of Gaucher's disease in this family had normal children. One of the suspected individuals had produced no live children but one stillborn baby and two abortions. The 'reproduction balance' in this family was: From 8 normal individuals in the second generation sprang 5 normal children and 2 abortions, from 2 patients plus 2 suspected cases sprang 2 normal children, one stillborn baby and two abortions, so that in the third generation there was no case of Gaucher's disease left.

#### FAMILY J (FIG 5)

Two Jewish girls, sisters (A X and E X) born in 1913 and 1927, respectively, were examined in 1939. A X had an enlarged spleen and liver, malar flushes, hypochromic anemia, leukopenia, thrombopenia, hemorrhagic diathesis, bone involvement, and typical pigmentation. Typical Gaucher's cells were found in the marrow.

E X underwent splenectomy in 1930. Gaucher's cells were identified in the spleen and kerosin was isolated from the specimen. She had swelling of the liver, transient pigmentation, malar flushes, bone involvement, anemia, thrombopenia, but no leukopenia. Gaucher's cells were present in the bone marrow.

The mother of these girls (I 2) was found to be normal on clinical examination. Her sternal marrow was normal. The father (I 1) had bilateral pingueculae and a few scattered gray spots on the skin of the legs. His liver and spleen were not enlarged. The blood picture was also normal. In the sternal marrow,

a few abnormal cells were found that might have been called reticulum cells or stem cells but the faintly-stained protoplasm was nonhomogeneous in character, and resembled in its structure compressed tissue paper. It was felt that these were actually small Gaucher cells and a diagnosis of subclinical Gaucher's disease was established. No other case was found in the family, although three brothers of the patients were examined clinically and by sternal puncture.

This then was a family in which, what seemed to be at first the sporadic occurrence of Gaucher's disease in two sisters could be explained by unmasking the father as a subclinical case or an almost normal 'carrier'. It seems significant that whereas this man had no clinical evidence of the disease at the age of 39, both his daughters were already affected as children and showed signs of steady progress of the disease.\*

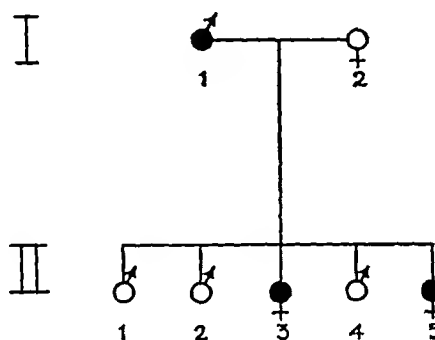


FIG 5 FAMILY J

#### DISCUSSION

The familial occurrence of Gaucher's disease without clear-cut transmission in a vertical line from parents to children has puzzled many observers. It has given rise to peculiar theories which challenge the established laws of heredity. Rowland,<sup>16</sup> for instance, writes: "As Gaucher's disease is a constitutional anomaly, that is, a mutation in the human species that follows Mendelian law, it is sometimes inherited recessively and sometimes shows dominant characteristics."

The concept of Gaucher's disease as a mutation is founded on its being a congenital anomaly of a sudden occurrence. However, a mutation, once it has established itself, would be expected to be transmitted as a constant entity from parents to offspring. Actually, very few instances have been observed in which a patient with Gaucher's disease has transmitted the disease to his offspring. This is, in the first place, due to the fact that many cases of Gaucher's disease occur in children who die before they reach the reproductive age. Among the cases that reach maturity, a number remain unmarried and among the married patients there is a remarkably high incidence of abortion, stillbirth, and of children who die shortly after birth. For the rest, a certain number of normal children have been observed among the offspring of patients with Gaucher's disease. This, however, cannot be an argument against the hereditary character since, even if a disease is dominant, it will affect only 50 per cent of the offspring. All these points, evident from the literature, were confirmed by the genetic data here presented.

\* The cases belonging to the families F to J have been described by Gerrer and Groen.<sup>9</sup>



Obviously, Gaucher's disease has a tendency to extinguish itself. If the disease is present at birth, death seems to result within a year.<sup>12</sup> If it begins in childhood, death occurs in the first or second decade. In young adults the disease can be compatible with life for many years, but the affected offspring die before or shortly after birth and only the unaffected children survive. The only cases, therefore, which are left to study the transmission of Gaucher's disease from a parent to his children are those in whom the disease manifests itself late in life.

An example of this situation is furnished by Family C. The father was a very mild case. When he was 45, he apparently had no clinical evidence of Gaucher's disease and the abnormality was discovered only when he was 65 years old. In his case, the disease had been so mild that it had not interfered with his reaching an advanced age and it had apparently permitted transmission to two children who survived. The transmission in this family was of a simple dominant type. It is significant that whereas the father developed clinical manifestations of the disease at the age of 65, both sons had already showed a marked enlargement of the spleen at the ages of 30 and 27, respectively. Apparently the disease became more serious in the second generation.\* This suggests that the frequent occurrence of abortion and stillbirth in the offspring of patients with Gaucher's disease may be due to the fact that the disease makes its appearance in the affected children at an age so early that it has a lethal effect, either during the intrauterine development or shortly thereafter.

Family B shows another interesting aspect of the hereditary mechanism. Here the disease was present in a number of *cousins* of the same generation. It seems an inevitable conclusion that the affected members of this family inherited their disease from the only ancestor they had in common. It so happened that they sprang from different grandmothers but from the same grandfather. This grandfather, therefore, must have had the genes abnormality.

We are forced to conclude that he transmitted the disease to his sons, who fathered the affected siblings. It is interesting that neither this grandfather nor his two sons exhibited any sign of Gaucher's disease during life, and as we have just concluded that the disease is dominant, the only explanation left is that Gaucher's disease might be present in a carrier individual in a mild, subclinical form. Furthermore one gets the impression from the data available that after every transmission the disease increases in severity until after two (or possibly more) generations, the anomaly is so severe that it manifests itself clinically. From then onwards, it would depend on the severity already reached whether one more affected individual can be produced which can still survive. In the end, however, the mutation becomes incompatible with reproduction or life, and thereby extinguishes itself. The high number of miscarriages, stillbirths and early deaths among the members of Family B is in harmony with this hypothesis.

\* This statement was strikingly demonstrated in the study of a recent case of severe Gaucher's disease in a boy of 16. His parents were apparently in excellent health, but examination of both of them revealed splenomegaly in the mother, both by palpation and by x-ray examination. The blood showed slight leukopenia, and the sternal marrow aspiration showed numerous Gaucher cells. X-rays of the bones were negative. This woman must be defined as a carrier since she had always been well and at the age of 4- appeared to be in excellent physical condition. *Editor*

A proof for this concept could be found in Family J. Here two girls were affected at an early age. The parents were seemingly normal, but the discovery of early Gaucher's cells in the bone marrow of the father unmasked him as a 'subclinical case' who acted as a carrier of Gaucher's disease. In Family I this proof could not be furnished because none of the parents was alive when the family was studied.

Several further points emerge from this hypothesis. It is possible, e.g., that what are commonly regarded as so-called 'sporadic' cases of Gaucher's disease may be individuals who acquired the disease straightway in a serious degree as the first mutation. It is also possible however, that many of these only manifest a disturbance which was already present in a subclinical degree in one of their parents who acted as a carrier.

Apart from the peculiarity that the disease tends to increase in severity in succeeding generations, the mechanism of transmission in Gaucher's disease appears to be following the genetic rules for one simple dominant trait. Strictly speaking, one would expect that in that case 50 per cent of the offspring would be affected. Actually, the number of siblings in the families described above is too small to decide this point. The patient in Family A had two children, both of whom died shortly after birth and were presumably affected. Two of the three siblings of the patient in Family C had the disease. In these two families the incidence of Gaucher's disease among the offspring seemed higher than 50 per cent. In Family I about half of the patient's offspring were either abortions or stillborns. In the Families B and J, however, the incidence of affected individuals among the offspring of the patients was less than one-half of the siblings and the patients F and H had one child each, neither of whom were affected. The total 'reproductive balance' of all the families was about as follows. Of 18 adult patients (including the two suspected cases from Family I) 9 were unmarried and 4 were married but had no children, giving an incidence of infertility of 72 per cent. The remaining 5 patients produced 2 abortions, 4 stillborn babies, 4 individuals with Gaucher's disease and 16 normal individuals. The incidence of affected or stunted individuals among the offspring of the Gaucher cases was therefore almost 40 per cent. A theoretic figure of 50 per cent, to be expected if Gaucher's disease were inherited as a simple dominant trait, might be reached if a larger number of families were investigated but in view of the high incidence of infertility it is doubtful if this figure would actually give a true picture of the situation. Among 15 normal members of the same generation as the patients, who reached the reproductive age and of whom we examined the offspring we found only 2 who were unmarried and 2 who were married but had no children. The incidence of infertility among this control group was 26 per cent. The remaining 9 persons produced 2 abortions, no stillborn babies, no cases of Gaucher's disease and 20 normal individuals. The incidence of stunted individuals among this group was only 9 per cent.

One of the important results of the present investigation is the demonstration of the subclinical or carrier state in the father of Family J by examination of the sternal marrow. This makes it imperative to use this technic in every genetic investigation of Gaucher's disease. Unfortunately shortly after this was realized the German invasion of the Netherlands occurred and almost all of the Jewish persons

described in this paper were seized and deported to Poland where they were exterminated. As a result the author was unable to perform sternal punctures on the seemingly normal individuals in these families. It is hoped that others who have an opportunity to carry out similar investigations will complement their clinical studies with marrow puncture. Whether healthy individuals in whom early Gaucher cells are found by this technic should best be named "subclinical cases" or 'carriers' is a matter of philosophy.

The substrate of Gaucher's disease is a disturbance in the metabolism of a special lipid, kersasin. It is commonly assumed that this lipid is a product of metabolism somewhere in the body which cannot be destroyed by the lack of some enzyme and is consequently stored in the reticulum cells. Another possibility is that there is an increased production of the substance inside these cells themselves.<sup>10</sup> However this may be, the seriousness of the disease depends on the total excess of kersasin that is present in the body. A small quantity of this substance inside the reticulum cells of spleen, liver, bone marrow, etc. does not result in appreciable harm. It is only when excessive amounts are present that the liver and spleen become enlarged, destruction of bone marrow occurs and a serious condition arises. It seems a likely supposition that Gaucher's disease is due to a deficiency of some specific enzyme. The seriousness of the disease would depend upon whether the enzyme is only somewhat diminished in quantity or totally lacking. It seems that as the disease is transmitted, the quantity of this hypothetical enzyme diminishes in the next individual. This concept is in harmony with what we see in many other hereditary diseases, where what is merely an innocent trait in a parent becomes an actual disease in the offspring.<sup>13</sup> Gaucher's disease is also another example indicating that hereditary diseases can occur in varying degrees of severity just as acquired diseases, a concept which many physicians still realize insufficiently.

Finally, attention should be drawn to the frequent occurrence of cholelithiasis and mild diabetes in the families with Gaucher's disease, both in the affected and in the normal siblings.

#### SUMMARY

The author reviews the literature of the familial incidence of Gaucher's disease. Almost all the familial cases which have been described occurred in the members of one generation (siblings or cousins) only. To these cases reported in the literature the author adds 25, of which 4 were 'sporadic' cases. The other 21 cases occurred in 6 families. The pedigrees of these families are presented. After an analysis of the available data the author presents the following hypothesis for the hereditary mechanism in these families.

Gaucher's disease is a mutation which, once established, is transmitted as a simple dominant hereditary trait. In the affected individuals this trait gives rise to a disturbance of lipid metabolism which results in the accumulation of kersasin in the reticulum cells throughout the body. The severity of the disease may vary considerably. It can be present in such a slight degree that the amount of kersasin accumulated during life is too small to give rise to clinical manifestations. In other cases the progression may be so slow that the disease becomes manifest only in

old age, provided the affected individual lives long enough. In these "subclinical cases," a diagnosis of Gaucher's disease can sometimes be made by the detection of 'early Gaucher cells' in the sternal marrow. Individuals thus affected suffer from Gaucher's trait rather than from the actual disease. However, they can transmit the disease to 50 per cent of their offspring, and thus function as (almost) normal carriers. In the family trees presented, it appeared that the disease tended to become more severe in every succeeding generation until after two or three generations it became clinically manifest in the affected individuals early in life. In the next generation it would then establish itself during fetal life so as to give rise to abortion, stillbirth or early death of the affected infant. In this way the mutation extinguishes itself, by permitting only the unaffected offspring of the affected individuals to persist. As a practical conclusion it is urged that a sternal marrow examination be included in every genetic investigation of Gaucher's disease as the best method available at present for the detection of subclinical cases or 'carriers'.

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# STUDIES CONCERNING THE PATHOGENESIS OF GAUCHER'S DISEASE

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S J THANNHAUSER, M D

IN 1882 Charles Ernst Gaucher<sup>1</sup> described as 'epithelioma primitif de la rate' the disease which now bears his name. In his case he found the splenic pulp entirely replaced by large cells and attributed this condition to a primary tumorous growth, epithelioma of the spleen. Collier<sup>2</sup> (1895) in England, and Picou and Ramond<sup>3</sup> in France (1896) also regarded the condition as neoplastic. Bovard<sup>4</sup> (1900), reporting the first case in this country, called attention to the simultaneous appearance of these large cells in the liver as well as in the spleen and lymph nodes. He had commented on the familial character of the disease. In contrast to the current view that the disease was of tumorous nature, he believed that an unknown toxin caused hyperplasia of spleen, liver, and lymph nodes. Brill, Mandlebaum and Libman<sup>5</sup> (1905) were the first to point out that the cells which characterized the disease were found, not only in the liver, spleen and lymph nodes, but appeared simultaneously in different parts of the skeleton. These authors suggested (1913) the name, 'Gaucher's disease,' to avoid the misleading term 'primary idiopathic spleno-megaly.' Schlagenhauser<sup>6</sup> (1906) considered that the condition was a systemic disease of the lymphhemapoietic tissue.

H. Lieb<sup>7, 8</sup> (1924, 1925), in association with Epstein and Lorenz,<sup>9</sup> isolated the substance which characterized the Gaucher cells and identified this substance as a cerebroside, namely kersasin. It was believed that kersasin, a galactosidocerebroside, was a constituent only of brain tissue and not of visceral organs. L. Pick<sup>10</sup> assumed that kersasin originated as a result of a general disturbance of intermediary lipid metabolism, accumulated in the blood, and was secondarily deposited and stored in the reticulum cells of the involved organs. He characterized Gaucher's disease 'not as a reticulo-endothelial, reticular cell or histiocytomatotic disease, but as a histiocytic disease comparable to histiocytic storage as observed in vital staining or cholesterol feeding of animals with, however, elective participation of certain histiocytic forms.'

In contrast to the conception of L. Pick,<sup>10</sup> S. J. Thannhauser and co-workers<sup>11</sup> demonstrated that normal serum or serum of patients with Gaucher's disease did not contain measurable amounts of cerebroside. This observation was subsequently confirmed by Dvoracek and Pest,<sup>12</sup> and Bruckner.<sup>13</sup> Since cerebroside is not present in the serum of Gaucher's disease, Thannhauser<sup>14</sup> concluded that cerebroside does not originate as a result of a general disturbance of the intermediary lipid metabolism but is synthesized and stored in the cells where they are found, i.e., in the Gaucher cells. This explanation of the pathologic formation of cerebroside places the metabolic disorder within the reticulum cells and histiocytes.

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An imbalance of the intracellular enzymes concerned with cerebroside formation and disintegration is assumed to be the fundamental defect in Gaucher's disease. Similarly in Niemann-Pick's disease an enzymatic intracellular disturbance presumably leads to increased sphingomyelin formation. An enzymatic deviation of the intracellular lipid metabolism is therefore considered as the etiology of these disorders.

This view is supported by the experiments of Thannhauser and Reichel<sup>15</sup> and by Ottenstein, Schmidt and Thannhauser,<sup>16</sup> who demonstrated that cerebrosidase is an intracellular enzyme which cannot be extracted from the tissue.

A further support for the view that the cerebroside in Gaucher's disease are synthesized and stored in the cells where they are found is evident from the discovery of an hitherto unknown group of cerebroside occurring exclusively in the Gaucher cells, namely a glucosidocerebroside (Halliday, Deuel, Tragerman and Ward<sup>17</sup> and Aghion<sup>18</sup>). In glucosidocerebroside, the carbohydrate group is glucose and not galactose as in kerafin. The findings of Halliday and co-workers have been confirmed by Klenk,<sup>19</sup> Danielson, Hall and Everett<sup>20</sup> and by Polonovski.<sup>21</sup>

The question arises whether in Gaucher's disease an abnormal cerebroside, i.e., glucosidocerebroside, is formed exclusively or whether in different individual cases the type of cerebroside may vary and both cerebroside, galactosido- (kerafin) and glucosidocerebroside may be present together.

The purpose of this paper is an attempt to decide this question by a method that permits quantitative partition of cerebroside into galactosido- and glucosidocerebroside. This method is described in the present paper and applied to the analysis of organs of different cases of Gaucher's disease. It will be demonstrated that in individual cases the organs vary in the amount and type of cerebroside present.

Our investigations were carried out with the additional purpose of clarifying another question regarding the pathogenesis of Gaucher's disease. It is known that normal red blood cells contain a small amount of cerebroside. It might be inferred that the increase of cerebroside in Gaucher organs may have resulted from a primary accumulation of cerebroside in the red blood cells. Deposition of this material in the organs in Gaucher's disease could occur even though the serum did not contain appreciable amounts of this lipid.

It will be shown by the chemical analysis of red blood cells from normal individuals and from patients with Gaucher's disease that the cerebroside present are quantitatively and qualitatively the same. These findings seem to support the conception of Thannhauser and co-workers<sup>11-14</sup> that the large amount of cerebroside present in the organs involved in Gaucher's disease is due to their synthesis and storage in the cells where they are found.

## MICRODETERMINATION OF CEREBROSIDES IN RED BLOOD CELLS AND IN TISSUES

### PRINCIPLE

The characteristic group which distinguishes the cerebroside from most of the other lipids is their carbohydrate component. Since the carbohydrate group can be liberated quantitatively from the cerebroside by acid hydrolysis the estimation of the free sugars in hydrolysate total of the lipid fraction is at present the basis of all analytical procedures for the quantitative determination of cerebroside.

(Brückner,<sup>13a, b</sup> Halliday, Deuel, Tragerman and Ward,<sup>17</sup> Aghion,<sup>18</sup> Klenk,<sup>19</sup> Danielson, Hall and Everett,<sup>2</sup> Polonovski,<sup>20</sup> and Brand and Sperry<sup>21</sup>)

The conversion of the results of the sugar determination into cerebroside values, however, requires the application of certain precautions

1 The crude alcoholic lipid extract contains in addition to the cerebroside noticeable amounts of nonlipid carbohydrates. The removal of the contaminating carbohydrates is achieved by extracting the final solutions of the lipids in chloroform repeatedly with an aqueous solution of trichloroacetic acid

2 It was found by Brand and Sperry,<sup>22</sup> as well as by us, that the customary hydrolysis of the lipids with sulfuric acid entails considerable losses due to destruction of carbohydrates, but that hydrochloric acid under certain conditions permits the quantitative recovery of the sugars

3 It has recently been shown that the carbohydrate component of the cerebroside is not exclusively galactose, but at least under certain conditions in Gaucher's disease,<sup>17-19</sup> a mixture of galactose and glucose is present. Since equal amounts of galactose and glucose have different reduction values, the correct conversion factor can only be established by separate determination of glucose and galactose in the hydrolysate. Our procedure for the quantitative partition of the sugars into galactose and glucose is based on the removal of the latter by yeast fermentation

### PROCEDURE

**Extraction** The amount of material required for the determination varies according to its cerebroside content. The following amounts of various tissues have been found to be suitable for the determinations of cerebroside: 10 Gm. of washed red blood cells corresponding to approximately 40 cc. of oxalated blood, 5 to 10 Gm. of wet brain, 20 Gm. of visceral organs, 50 mg. of crystallized cerebroside mixtures. For the quantitative extraction of cerebroside the material is homogenized in the Waring Blendor with 20 to 30 volumes of 95 per cent alcohol. The suspension is refluxed for 30 minutes and filtered while still hot. The residue is re-extracted by refluxing with the same amount of 95 per cent alcohol. The combined extracts are evaporated to dryness under reduced pressure. The oily residue is refluxed with a mixture of 4 volumes of chloroform and 1 volume of ethanol, filtered while still hot and washed with the chloroform-ethanol mixture.

**Removal of nonlipid carbohydrates** The combined extracts and washings are transferred to a 250 cc. centrifuge tube and stirred mechanically with four volumes of 2 per cent trichloroacetic acid for ten minutes. After separation of the emulsion by centrifugation the aqueous top layer is siphoned off and discarded. The extraction with trichloroacetic acid is repeated at least twice until a microdetermination of the sugar (Somogyi<sup>23</sup>) in an aliquot of the aqueous layer shows the absence of reducing carbohydrates.

**Hydrolysis** The chloroform-ethanol extract is transferred to a 60 cc. centrifuge tube and brought to dryness at room temperature by an air current which is bubbled through the solution. The residue is suspended in 15 cc. of an aqueous, 1:5 N, solution of hydrochloric acid by means of a glass rod. The hydrolysis is carried out on a boiling water bath for sixty minutes. The centrifuge tube is covered with a loosely fitting glass bulb. The cooled hydrolysate is brought to a volume of 25 cc., sharply centrifuged and filtered.

**Removal of free acid by Amberlite** Prior to the sugar determination, the free acid is removed from the hydrolysate by absorption on Amberlite (analytical grade IR 4B). (The neutralization cannot be carried out by the addition of alkali since the presence of salts interferes with the subsequent fermentation.) For this purpose an aliquot of the hydrolysate is stirred mechanically with the resin (2 Gm. per 10 cc. hydrolysate\*), for twenty minutes until the color of congo red paper is not changed by a drop of the supernatant.

All controls are brought to a volume of 10 cc. and incubated for three hours at room temperature. After centrifugation, the reduction is determined in an aliquot of the supernatant according to Somogyi.<sup>23</sup> The mixture is brought to a volume of 10 cc. in a volumetric flask and fermented at room temperature for 3 hours. After centrifugation, the nonfermentable sugar is determined in 5 cc. of the supernatant according to Somogyi.<sup>23</sup>

\* Control experiments with known glucose solutions have shown that analytical grade Amberlite does not absorb any glucose.

In a second aliquot of 4 cc of the Amberlite supernatant the total carbohydrates are determined according to Somogyi without fermentation

*Calculation* The titration value F obtained in the fermented aliquot represents galactose and is accordingly multiplied by the factor 0.20 in order to obtain the amount of galactose in milligrams. The difference between F and between the titration value T obtained with the unfermented aliquot represents glucose and is multiplied by the factor 0.14 (Somogyi)

In order to calculate the amount of cerebrosides the galactose, respectively the glucose values are multiplied by the factor 4.6

*Determination of galactose and of total carbohydrates* An aliquot of 8 cc of the Amberlite supernatant is mixed with 1 cc of a 10 per cent suspension of fresh baker's yeast\* (Fleischmann)

Analyses of the cerebrosides in normal serum and Gaucher serum showed

<i>Normal serum</i>		<i>Gaucher serum</i>	
Galactosidocerebrosides	Glucosidocerebrosides	Galactosidocerebrosides	Glucosidocerebrosides
Traces	0	Traces	0

The analyses were made on 30 cc samples of serum of three normal individuals, as well as on 30 cc samples of two patients with Gaucher's disease (case Ke, 14 years old and case Le, 24 years old)

These figures confirm the analytical findings of Thannhauser and co-workers,<sup>11</sup> Brückner,<sup>12a</sup> Dovracek and Pesta<sup>12</sup> indicating that the serum of Gaucher's disease does not contain measurable amounts of cerebrosides

TABLE 1 — ANALYSIS OF RED BLOOD CELLS IN NORMAL INDIVIDUALS AND PATIENTS WITH GAUCHER'S DISEASE

Normal R B C Per cent of desiccator dried weight		Gaucher R B C Per cent of desiccator dried weight	
Galactosidocerebrosides	Glucosidocerebrosides	Galactosidocerebrosides	Glucosidocerebrosides
0.189	0	0.200 (case Ke) 0.220 (case Le)	0 (case Ke) 0 (case Le)

The analyses of red blood cells, washed with saline, were made in three normal individuals from an average of 9.8 Gm of dried material, the samples being dried in a desiccator at room temperature

The analyses of Gaucher red blood cells, washed with saline, were made in case Ke, 14 years old, on 7.7 Gm dried substance, in case Le, 24 years old, on 8.6 Gm dried substance

The figures in table 1 demonstrate that the content and the type of cerebroside in the red cells of Gaucher's disease do not differ from those found in normal red blood cells. It is important to note that both in the normal and Gaucher's disease, only galactosidocerebrosides were found in the red cells even when glucosidocerebrosides were accumulated in the Gaucher cells of the spleen, as in case Ke (corresponding to Case 1 in table 2)

The organs of cases 1 and 2 were from children of 6 to 10 years of age, cases 3 and

\* With each series of analyses the following additional control determinations are carried out: (a) 1 cc of the washed yeast suspension is mixed with water; (b) 1 cc of the washed yeast suspension is mixed with a known amount of glucose; (c) 1 cc of the washed yeast suspension is mixed with a known amount of galactose



TABLE 2.—ANALYSES OF SPLEENS IN 4 CASES OF GAUCHER'S DISEASE BY ISOLATION

Crystalline cerebrosidcs from Gaucher spleen	Total cerebrosidcs in per cent of dry weight	Galactosido-cerebrosidcs in per cent of dry weight	Glucosidocercbrosidcs in per cent of dry weight	Ratio galactocercbrosidcs glucosidocercbrosidcs
Gaucher spleen 1	8.3	Negligible	8.3	0.83
Gaucher spleen 2	11.3	Negligible	11.3	0.113
Gaucher spleen 3	15.8	8.6	7.2	1.21
Gaucher spleen 4* (See below)	21.6	12.0	9.6	1.31

Case 4—Analyses by Analytic Determination

	Total cerebrosidcs in Gm per 100 Gm tissue	Galactosido-cerebrosidcs in Gm per 100 Gm tissue	Glucosidocercbrosidcs in Gm per 100 Gm tissue	Ratio galactocercbrosidcs glucosidocercbrosidcs
Gaucher spleen (Tissue)	29.4	14.4	15.0	1.1
Normal spleen (Tissue)	0.4-0.6	0.4-0.6	Negligible	—

\* In case 4 the galactosidocerebrosidcs and glucosidocerebrosidcs were also determined directly in the organ

TABLE 3.—Case 1 Higgins, E., aged 7 months

Diagnosis Generalized Infantile Gaucher's Disease Organs Preserved in Formaldehyde, Received October, 1945, from Dr. Robb-Smith, Radcliffe Infirmary, Department of Pathology, Oxford, England

Organ	Chemical analysis		
	Galactocerebrosidcs Gm per cent of dry weight	Glucocerebrosidcs Gm per cent of dry weight	Galactocerebrosidcs in normal human organs Gm per cent of dry weight
Brain	2.6	Negligible	4.0-6.0 (1.0)*
Spleen	3.2	"	0.1-0.5
Liver	2.3	"	0.05-0.15
Lungs	6.8	"	0.1-0.6
Heart	2.3	"	0.24
Bonemarrow	1.3	"	—
Kidney	3.1	"	0.1-0.7
Intestine I	3.2	"	—
Intestine II	2.8	"	—
Pancreas	3.1	"	—

\* Cerebrosidcs in the brain of a one day old child

4 from adults. In cases 1, 2, and 3 the cerebrosidcs were isolated in crystalline form and the analyses were made for galactosido- and glucosidocerebrosidcs.

In case 4\* the galactosidocerebrosidcs and glucosidocerebrosidcs were determined

\* We are indebted to Dr. Karl Singer, Michael Reese Hospital, Chicago, for sending this spleen of an adult Gaucher's disease

directly in the organ as well as after isolation of the crystalline cerebrosides mixture

The analytic figures of normal human and animal spleen are charted in table 2

The figures in table 3 and table 4 demonstrate that even in the organs of siblings with generalized infantile Gaucher's disease the amount and the type of galactosido- and glucosidocerebrosides may vary greatly. The intracellular metabolic disorder in Gaucher's disease is thus not solely confined to the formation of an abnormal glucosidocerebroside, since in one of the examined siblings, mainly galactosidocerebrosides and in the other sibling galactosido- and glucosidocerebrosides in varying amounts were found in the organs

TABLE 4—Case 2 Higgins, Timothy, aged 5½ months (Sibling of Case 1)

Diagnosis Generalized Infantile Gaucher's Disease (Organs received from Dr Robb-Smith, Radcliffe Infirmary, Oxford, England)

Organ	Chemical Analysis				
	Galactocerebrosides per cent of dry weight	Glucocerebrosides per cent of dry weight	Ratio galactocerebrosides to glucocerebrosides	Total cerebrosides per cent of dry weight	Galactocerebrosides in normal human organs per cent of dry weight
Spleen	0.44	0.95	1:2:1	1.44	0.1-0.5
Liver	0.28	0.05	5:6:1	0.33	0.05-0.15
Brain	3.25	0		3.25	4.0-6.0*
Lung	0.61	0.43	1:4:1	1.04	0.1-0.6
Kidney	0.29	0.25	1:2:1	0.54	0.1-0.7
Pancreas	0.09	0.05	1:8:1	0.14	0.1-0.3
Thymus	0.30	0.30	1:0:1	0.60	—
Suprarenal	0.20	0.09	2:2:1	0.29	—

\* Cerebrosides in the brain of a one day old child

### DISCUSSION

The above findings (table 2) demonstrate that the spleen of two children with Gaucher's disease contained glucosidocerebrosides almost exclusively, thus confirming the observations of Halliday, Deuel, Tragerman and Ward,<sup>17</sup> Aghion,<sup>18</sup> Klenk,<sup>19</sup> Danielson, Hall and Everett,<sup>20</sup> and Polonovski.<sup>21</sup>

In cases 3 and 4, however, both galactosido- and glucosidocerebrosides were present. These findings are the first demonstration that both types of cerebrosides may be increased in the organs of patients with Gaucher's disease.

The fact that the galactosidocerebrosides and the glucosidocerebrosides may be found together indicates that the increased formation of cerebrosides within the Gaucher cells is not a complete deviation from the synthesis of the normal galactosidocerebrosides. The occurrence of the abnormal glucosidocerebrosides exclusively within the Gaucher cells lends support to the theory that these cerebrosides are built and stored in the Gaucher cells.

The alternating occurrence of galacto- and glucosidocerebrosides is especially evident in the cases of 2 siblings with generalized infantile Gaucher's disease. In the 7 months old infant, mainly galactosidocerebrosides were present with only

traces of glucosidocerebrosides, while in the  $5\frac{1}{2}$  months old infant both types were increased in all organs examined. It is probable that there is no fundamental difference in the chemical intracellular disorder of the generalized infantile and of the adult Gaucher's disease.

It was formerly believed that the disordered enzymatic metabolism occurred in the *milieu interieur* of the organism and was the result of the enzyme content of the secretions of glandular structure. The concept that enzymes exist bound to the cellular structure is relatively new (Desmoenzymes Willstaetter).

Embryonic reticulum cells and histiocytes have apparently a greater variety of enzymatic possibilities than mature cells. This seems especially true in the case

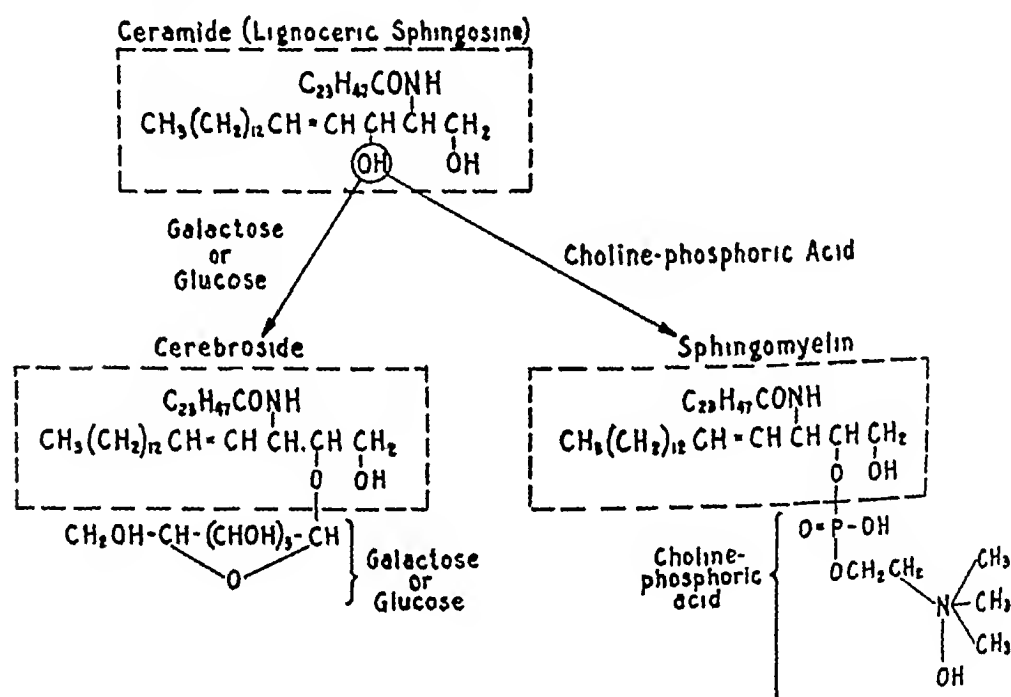


FIG. 1.—CEREBROSIDE AND SPHINGOMYELIN FORMATION FROM CERAMIDE

of the lipid substances such as cerebroside, sphingomyelin and cholesterol. In Gaucher's disease the capacity to synthesize cerebroside in increased amounts is, in our opinion, a perpetuation of the embryonal enzymatic potentialities of certain reticulum cells and histiocytes. The suggested physiology and pathology of the enzymatic metabolism of cerebroside and sphingomyelin is pictured in figure 1.

The ceramides isolated and identified by Thannhauser and Frankel<sup>24</sup> are common intermediaries of the metabolism of both cerebroside and sphingomyelin. Enzymatic glucosido-formation with glucose or galactose leads to the formation of cerebroside, while sphingomyelin results by esterification with phosphorylcholine. In the case of Gaucher's disease cerebroside is formed apparently from the ceramides in abundance, while the intermediate disintegration of cerebroside to ceramide is diminished.

## SUMMARY

1 The serum of normal individuals and of patients with Gaucher's disease does not contain cerebroside in measurable amounts. Cerebroside in Gaucher's disease are increased only in those organs containing abundant numbers of cells characteristic of the disease.

2 Normal red blood cells contain approximately 0.19 per cent cerebroside. The cerebroside in red blood cells is a galactosidocerebroside.

3 Red blood cells in Gaucher's disease do not differ quantitatively and qualitatively in their cerebroside content from the red blood cells of normal individuals.

4 In four different cases of Gaucher's disease separate determinations of splenic galactosido- and glucosidocerebroside were made. The spleen of two adults showed mainly glucosidocerebroside and only traces of galactosidocerebroside, while the analysis of the spleen of two other adults showed that galactosido- as well as glucosidocerebroside may be accumulated simultaneously in Gaucher cells. These findings are of importance, since it is demonstrated that the deviation of the cerebroside metabolism in Gaucher cells not only results in the formation of an abnormal glucosidocerebroside, but also may lead to the increased formation of the normal galactosidocerebroside, *kerasin*. It is demonstrated that the relative proportions of galactosido- and glucosidocerebroside in Gaucher cells may differ considerably in individual cases.

5 The organs of infantile siblings with generalized infantile Gaucher's disease were analyzed. The organs of one infant showed mainly galactosidocerebroside while in the organs of the other sibling both kinds of cerebroside, glucosido- as well as galactosidocerebroside, were present.

6 The findings reported in this paper lend support to the theory that Gaucher's disease is the result of a deviation of the intracellular metabolism of reticulum cells and histiocytes. The cerebroside is not transported by the serum or by the red blood cells and secondarily deposited in the cells involved, but is formed and stored in the reticulum cells and histiocytes where they are found.

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# EFFECTS OF X-RAY ON LYMPHOID AND HEMOPOIETIC TISSUES OF ALBINO MICE

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HEINEKE,<sup>1</sup> investigating effects of x-rays on internal organs, found the lymphoid tissues peculiarly susceptible to irradiation. This observation suggested the possibility of studying the function of the lymphoid apparatus by observing animals deprived of their lymphoid tissue by means of selective injury by x-ray.<sup>2</sup> For this purpose it is necessary to suppress lymphopoiesis without interfering with other bodily functions of the experimental animals used. Permanent destruction of the lymphoid tissues cannot be accomplished without deleterious effects upon other organs, as shown by Hughes and Job.<sup>3</sup> However, with a single whole body irradiation of 400 r, Henshaw<sup>4</sup> produced in albino mice marked temporary lymphopenia accompanied by only slight neutropenia and anemia. The dosage used by Henshaw thus appeared to produce the highly selective damage of lymphoid tissues necessary for functional studies of this system. The present study was undertaken to furnish further details of the selectivity of radiation injury produced with this dosage by measuring the initial damage to all blood-forming tissues and the reticulo-endothelial system, the extent and duration of the suppression of their activity, and the rate of their recovery.

## MATERIAL AND METHODS

Inbred female mice of the CFW strain, 2 months old, were given single whole body radiation of 400 r as measured in air with the Victoreen ionization chamber (186 KV, 20 Milliamps, at 50 cm with 0.25 mm copper and 0.55 mm aluminum filters, for 6.24 minutes). All animals survived the period of the experiment without obvious illness. The animals were examined in groups of six on the 1st, 2nd, 4th, 7th, 10th, 14th, 21st and 28th day after irradiation. On three mice of each group of six, peripheral white blood cell counts and differential counts were done, as well as hemoglobin determination with the Evelyn photoelectric colorimeter, standardized by Van Slyke's method. On the remaining three mice of each group the peripheral red blood cells and reticulocytes were counted and the hematocrit values determined using Van Allen's method. All six mice were then killed with chloroform. The animals were weighed and autopsies were performed. The superficial cervical lymph nodes, thymus, liver and spleen were weighed and fixed in Helly's fluid. Kidneys, heart and lungs were also fixed in Helly's fluid, but not weighed. The gastrointestinal and genital tracts were inspected for gross lesions. The brain and cord were not examined. Paraffin sections were prepared and stained routinely with hemalum azure eosinate and with hematoxylin-eosin. In addition the Rio-Hortega Foot silver reticulum stain, the Berlin blue reaction for iron, and the periodic acid-leukofuchsin stain<sup>9</sup> were used extensively on sections of the lymph nodes, spleen and thymus. Frozen sections of lymph nodes for the demonstration of fat were prepared in a few instances.

The bone marrow of one femur was used for the preparation of marrow smears.<sup>5</sup> Smears were routinely stained with Giemsa and frequently also with the peroxidase stain.<sup>6</sup> The other femur and several thoracic vertebrae were fixed in formalin and decalcified. Paraffin sections of them stained by van Gieson's method. Photomicrographs of the marrow of the epiphysis, metaphysis and shaft of the femur and of one vertebra were then prepared. Each photomicrograph was superimposed upon a cross ruled transparent grill. The

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number of squares of available marrow space and the number occupied by cellular marrow were each counted. The cellularity of the marrow in each area photographed was expressed as the percentage of available marrow space occupied by cellular marrow. The average of the 4 determinations for each animal gave the bone marrow index, which is defined<sup>7</sup> as the average percentage of available marrow space occupied by hematopoietic marrow. To obtain an index for each cell type, the total bone marrow index was multiplied by the percentage of individual cell types determined by differential counts of bone marrow smears prepared from the same animal. The basic concept of reporting bone marrow findings in this way is comparable to recording absolute counts of individual cell types in the peripheral blood rather than percentage figures of the total leukocyte count. Of necessity the standard of reference in the bone marrow is the available marrow space, while peripheral counts can be expressed in cells per cu. mm. The indices give a clear picture of the depletion of the marrow and the rate of subsequent regeneration of the various cell types. Bone marrow indices rather than differential counts are therefore used throughout this paper, with one exception. Subgroups of the myeloid series are reported as percentages of the total myeloid cells, as shifts to the left or right are meaningful with reference to the myeloid series only and not with reference to the available marrow space.

The classification of the granulocyte series in the bone marrow of small rodents presents certain difficulties, which have been discussed in an earlier paper by Endicott and Ott.<sup>7</sup> In the mouse as in the rat, maturation of the nuclear chromatin and of the cytoplasm of the granulocytes may proceed independently.

Young nuclei of round or oval shape with finely divided chromatin are frequently present in cells with old neutrophilic cytoplasm, while other cells show young basophilic cytoplasm and old ring form nuclei with coarsely distributed chromatin. This independent maturation of nucleus and cytoplasm makes any attempt to arrange the granulocytic series into definite promyelocyte, myelocyte and metamyelocyte stages highly artificial and subject to extreme variation from differences of interpretation. However, the very young and the definitely mature granulocytes are easily identifiable. For this reason the following classification was adopted:

*a Young forms* Relatively large cells with blue cytoplasm, with or without azurophilic granules, with round or early ring form nuclei, and finely divided nuclear chromatin.

*b Old forms* Cells with pale blue or neutrophilic cytoplasm, with ring form nuclei showing early indentation or fully developed segmentation and coarsely distributed chromatin.

*c Intermediate forms* All other cells of the granulocyte series, i.e., cells showing partial maturation of cytoplasm and nuclear chromatin in various combinations.

The above classification cannot be correlated exactly with the standard division of granulocytes into promyelocyte, myelocyte and metamyelocyte stages. It is felt that this disadvantage is outweighed by the ease with which differential counts on the same specimen can be reproduced by different observers. Any discrepancies can usually be traced to failure to take counts from at least 3 representative areas. The larger cells of the early granulocyte series and the small normoblasts tend to accumulate at opposite ends of the smears. For this reason, the beginning, middle and end portions of each smear were counted, going from the middle to one edge of the slide in each of these areas. Using this method, total cell counts usually comprise 800 to 1500 cells.

The average weights of the animals, the organ weights, peripheral blood counts and myelograms of the first series of mice killed in groups of six at the above stated intervals after irradiation are recorded in tables 1-4. Duplicate experiments were performed on a second series of animals of the same strain, sex and age and on a third series of mice of the same strain and sex, but 5 to 6 months old. The peripheral blood counts and myelograms for the latter two groups showed no significant deviation from the data of the first series recorded in our tables. Histologic findings were identical in all experiments. In a further small series of animals sections of lymph nodes, thymus, spleen, and bone marrow were examined 1, 2, 3, 7 and 18 hours after irradiation, but no blood counts or myelograms were taken.

Four groups of six control mice of the same strain and sex were examined at various intervals in the course of the experiment. The age of the animals was 2 months in 3 of these groups, and 5 months in the 4th group. In contrast to the narrow range of WBC counts in irradiated animals, individual WBC counts in the 24 control animals ranged from 5800 to 25,000. However, leukocyte counts of control animals in any one group of six tended to be on either the high or the low side, and fell within a relatively narrower range.

TABLE 1—*Body and Organ Weights (in Grams) of Mice Given Whole Body X-radiation of 400 r (Means and Standard Deviations)*

Days after x-ray	Mouse	Liver	Spleen	Thymus	Submaxillary lymph nodes
1	17.2 ± 2.1	1.21 ± 0.09	0.028 ± 0.004	0.022 ± 0.010	0.012 ± 0.003
2	15.6 ± 2.4	1.00 ± 0.17	0.012 ± 0.004	0.017 ± 0.011	0.006 ± 0.006
4	17.4 ± 4.6	0.95 ± 0.13	0.014 ± 0.004	0.008 ± 0.006	0.010 ± 0.003
7	17.7 ± 3.2	1.08 ± 0.21	0.021 ± 0.006	0.014 ± 0.006	0.019 ± 0.017
10	20.6 ± 3.2	1.06 ± 0.07	0.051 ± 0.022	0.029 ± 0.009	0.023 ± 0.007
14	17.2 ± 1.9	1.09 ± 0.14	0.110 ± 0.064	0.026 ± 0.009	0.039 ± 0.036
21	15.2 ± 1.8	1.02 ± 0.12	0.083 ± 0.014	0.013 ± 0.009	0.015 ± 0.008
28	15.5 ± 2.6	0.92 ± 0.20	0.102 ± 0.029	0.034 ± 0.016	0.023 ± 0.017
Control	16.8 ± 2.0	0.93 ± 0.14	0.095 ± 0.029	0.021 ± 0.008	0.023 ± 0.006

TABLE 2—*Bone Marrow Indices in Mice Given Whole Body X-radiation of 400 r (Means and Standard Deviations)*

Days after x ray	Percentage of available bone marrow space occupied by					
	Total marrow	Non-eosinophilic granulocytes	Eosinophils	Nucleated red	Lymphocytes	Others
1	49.6 ± 18.2	44.3 ± 15.2	1.6 ± 0.7	0.6 ± 0.4	2.0 ± 1.3	1.1 ± 1.2
2	22.8 ± 7.4	19.5 ± 8.4	0.5 ± 0.2	0.4 ± 0.2	2.0 ± 0.7	0.4 ± 0.2
4	16.8 ± 5.1	14.0 ± 3.2	Less than 0.1	0.1 ± 0.3	2.0 ± 1.3	0.7 ± 0.2
7	36.4 ± 11.1	24.1 ± 8.6	Less than 0.1	10.0 ± 7.4	1.5 ± 1.1	0.8 ± 0.4
10	64.6 ± 10.9	12.4 ± 11.1	0.8 ± 0.8	22.6 ± 8.0	26.3 ± 15.1	2.5 ± 1.3
14	67.0 ± 13.8	50.7 ± 19.1	0.3 ± 0.4	12.2 ± 5.2	2.8 ± 4.1	1.0 ± 0.5
21	91.4 ± 7.8	82.0 ± 6.8	0.9 ± 0.9	4.8 ± 4.1	1.8 ± 0.9	1.9 ± 0.4
28	94.6 ± 2.8	72.6 ± 12.5	2.8 ± 2.8	13.9 ± 8.3	4.0 ± 2.4	1.3 ± 1.3
Control	84.0 ± 9.6	43.9 ± 12.8	1.4 ± 1.1	21.4 ± 6.1	15.6 ± 3.5	1.7 ± 0.4

TABLE 3—*Differential Count of Noneosinophilic Granulocytes in Bone Marrow of Mice Given Whole Body X-radiation of 400 r (Means and Standard Deviations)*

Days after x ray	Young forms	Intermediate forms	Old forms
	%	%	%
1	18.3 ± 10.1	21.2 ± 5.9	60.5 ± 14.0
2	9.4 ± 3.9	19.8 ± 1.9	70.8 ± 4.6
4	14.0 ± 3.1	43.0 ± 6.6	43.0 ± 8.6
7	14.8 ± 2.3	38.1 ± 11.0	47.1 ± 12.2
10	56.8 ± 19.0	32.3 ± 9.0	11.0 ± 12.7
14	40.0 ± 9.4	37.4 ± 5.3	22.6 ± 9.2
21	23.7 ± 5.6	30.0 ± 3.8	46.3 ± 7.6
28	18.6 ± 7.4	33.5 ± 7.8	47.9 ± 9.9
Control	12.0 ± 3.4	32.9 ± 7.0	55.1 ± 8.5

For estimates of the degree of leukopenia in irradiated animals it is desirable to know the lower limit of the normal leukocyte count rather than the average count. For this reason the means and the standard deviations of the leukocyte count of each of the four groups of control mice were computed. The group



showing the lowest average leukocyte count is recorded as control group in our tables. The standard deviation of this group indicates that  $\frac{2}{3}$  of any group of animals with low normal counts may be expected to have lymphocyte counts of 5000 or more. Myelograms of all four control groups did not differ significantly.

TABLE 4—*Peripheral Blood Counts (WBC per cu mm) in Mice Given Whole Body X-radiation of 400 r (Means and Standard Deviations)*

Days after x ray	Total WBC	Lymphocytes	Neutrophils		Eosino phils
			Segmented	Juvenile	
1	3200 $\pm$ 700	2200 $\pm$ 200	900 $\pm$ 650	50 $\pm$ 50	50 $\pm$ 50
2	2100 $\pm$ 800	1200 $\pm$ 200	800 $\pm$ 700	50 $\pm$ 50	25 $\pm$ 25
4	880 $\pm$ 300	800 $\pm$ 300	25 $\pm$ 25	less than 25	less than 25
7	1100 $\pm$ 300	750 $\pm$ 200	300 $\pm$ 150	less than 25	less than 25
10	2100 $\pm$ 900	1700 $\pm$ 800	200 $\pm$ 100	200 $\pm$ 100	less than 25
14	4100 $\pm$ 400	2500 $\pm$ 100	700 $\pm$ 150	900 $\pm$ 300	50 $\pm$ 50
21	9000 $\pm$ 5200	5400 $\pm$ 2600	2600 $\pm$ 2400	900 $\pm$ 600	100 $\pm$ 150
28	8900 $\pm$ 800	6100 $\pm$ 500	2100 $\pm$ 600	400 $\pm$ 200	250 $\pm$ 250
Control	8500 $\pm$ 1300	6800 $\pm$ 1700	1400 $\pm$ 900	200 $\pm$ 120	100 $\pm$ 100

#### ORGAN WEIGHTS

The weight of the lymph nodes, spleen and thymus was considerably below normal 1, 2 and 4 days after irradiation. The weight of the thymus and the lymph nodes was again within normal limits 7 days after irradiation. The weight of the spleen was considerably increased over previous low values at 10 days, but did not reach normal until the 14th day after irradiation. The weights of all organs examined 21 and 28 days after irradiation did not differ significantly from those of the controls. The size of the lymphoid tissues observed at autopsies corresponded to the variations in weight recorded in table 1.

#### HISTOLOGIC FINDINGS

*Lymph nodes (controls)* Forty superficial cervical lymph nodes from 12 control mice, 2 months old, were studied as a basis for comparison with those of x-rayed mice. The capsule of the nodes is thin. As a rule there are no trabeculae. Medullary cords form the central core of the nodes and project in narrow bands toward the surface, dividing the cortical lymphoid tissue into ovoid masses of varying size. Follicles with more or less well developed secondary centers are present in greatly varying numbers. They are always located near the surface of the node, either embedded in a mass of cortical lymphoid tissue, from which they are demarcated by a rim of closely packed lymphocytes, or flanked by medullary cords.

The term cortical lymphoid tissue, as used here, is identical with the "substance corticale" of Jolly and the "Rindenknotten" of Hellman. In a loose network of reticulum cells and fibrils it contains free cells, mostly small lymphocytes, whose nuclei are frequently angular, indented or lobated, occasionally to an extent simulating a mitosis. Mitotic figures are rare in the cortical lymphoid tissue.

The follicles consist of medium sized and large lymphocytes, a few of which are seen in mitosis, and of a small number of large phagocytic cells. These contain

tingible bodies of Fleming, frequently clearly identifiable as disintegrating lymphocytes. A zone of closely packed, small lymphocytes surrounds the paler center. The term secondary center is used by us for this paler central zone, while the term secondary nodules is reserved for the entire follicle consisting of the paler center and the peripheral rim of closely packed lymphocytes. Reticulum is very scanty in the secondary centers.

The medullary cords consist of a central vessel surrounded by a concentric collar of large cells. These cells have a broad, slightly irregular rim of deeply basophilic cytoplasm and often somewhat eccentric nuclei of the lymphocytic type. They have been described as polyblasts and as plasmoidocytes.<sup>11,18</sup> Occasionally cells in mitosis are present. Plasma cells in varying numbers can usually be found in the medullary cords. The intermediary and medullary sinuses are sharply outlined by the marginal reticulum fibrils limiting the medullary cords.

The peripheral and intermediary sinuses are usually empty. A few medullary sinuses near the hilum frequently contain large numbers of polygonal or round cells, with an ample amount of oxyphilic cytoplasm. These probably represent desquamated lining cells of the sinuses. Mast cells in varying numbers are often present in the intermediary and medullary sinusoids. No appreciable amount of connective tissue is present in the hilum of the nodes.

*Lymph nodes of irradiated animals.* Sections taken 2 and 3 hours after irradiation showed widespread disintegration of lymphocytes. Large amounts of nuclear debris were present in macrophages in the secondary centers and occasionally in the cortical lymphoid tissue. At 7 hours cellular debris was also present in the medullary cords. At 18 hours hardly any lymphocytes remained in the secondary centers, which contained only swollen, poorly staining cells and large macrophages filled with cellular debris. The peripheral rim of closely packed lymphocytes, normally surrounding the secondary centers, was still well preserved in some of the follicles (fig. 1a). Other follicles had lost this collar of small lymphocytes and the former secondary centers, containing debris-laden macrophages, bordered directly on the peripheral sinuses (fig. 1b). Small accumulation of nuclear debris were still present in medullary cords and in the cortical lymphoid tissues. Presumably this debris was to a large extent ingested in macrophages, but the phagocytosing cells did not stand out clearly except in the secondary centers. A few granulocytes were occasionally present in the sinuses.

After 24 hours nuclear debris was seen only in macrophages in the remnants of the secondary centers. All follicles had now lost their peripheral rim of small lymphocytes. The medullary cords were partly collapsed and their cells often showed pyknotic nuclei. The medullary sinuses were correspondingly widened and the reticulum condensed (fig. 1c). Granulocytes were occasionally present in some of the sinuses.

The macrophages seen in the secondary centers at 7, 18 and 24 hours contained, in addition to nuclear debris, numerous small globules of oxyphilic cytoplasmic material, which stains vividly with periodic acid-leukofuchsin stain.<sup>19</sup> These globules were slightly acid-fast and slightly sudanophilic. Similar macrophages were sometimes also seen in the cortical lymphoid tissue and medullary cords. A

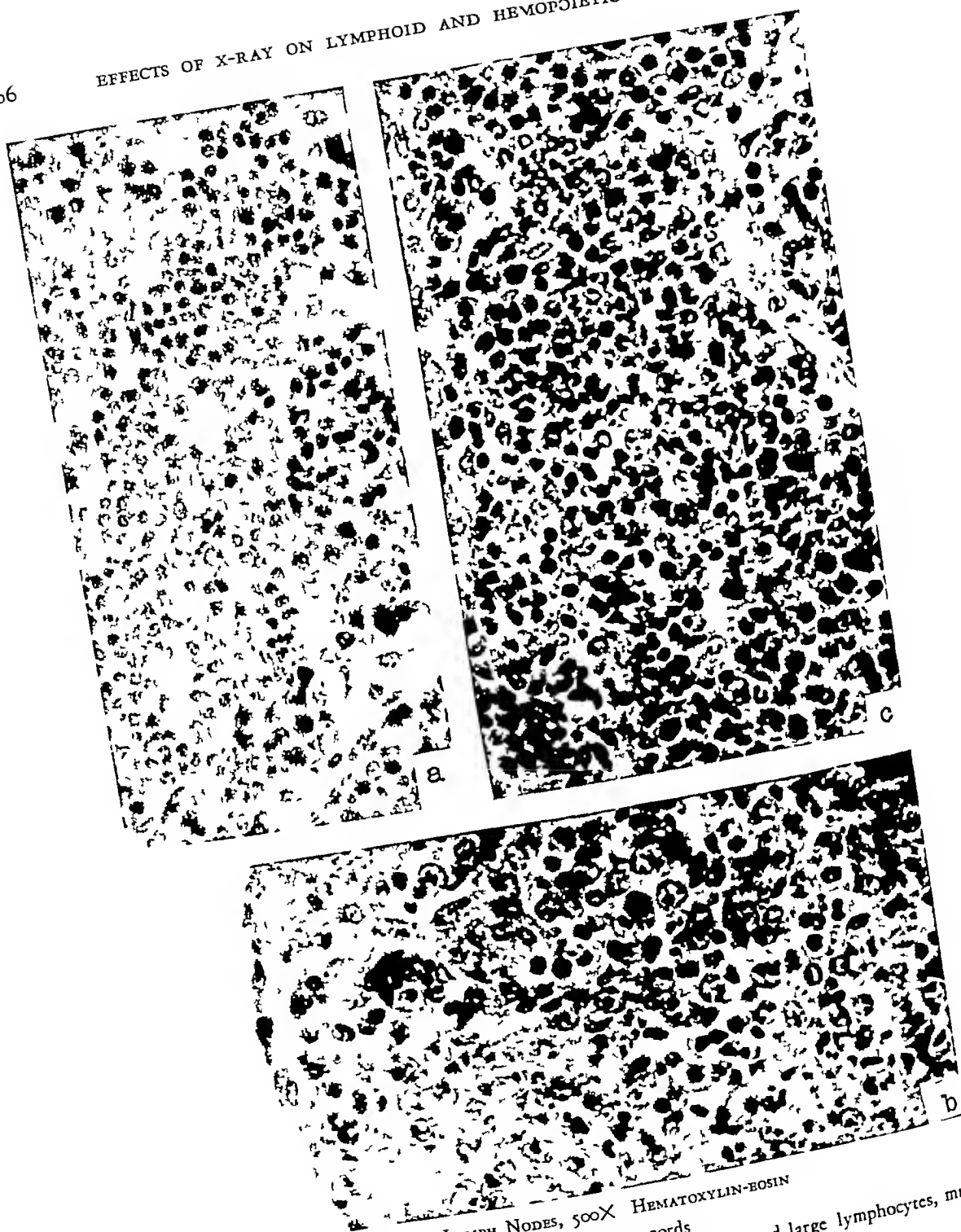


FIG. 2. LYMPH NODES, 500X. HEMATOXYLIN-EOSIN

- a 7 days after irradiation Lymphopoiesis in medullary cords
- b 10 days Portion of regenerating follicle showing medium sized and large lymphocytes, mitoses and phagocytosis absence of peripheral rim of small lymphocytes
- c 21 days Myelopoiesis in medullary cords

the cortex as compared with the medulla Lymphocytes were still present in reduced numbers in the central portion of the thymus This occasionally produced a

somewhat more cellular and darker appearance of the medulla as compared with the cortical areas (fig 3a)

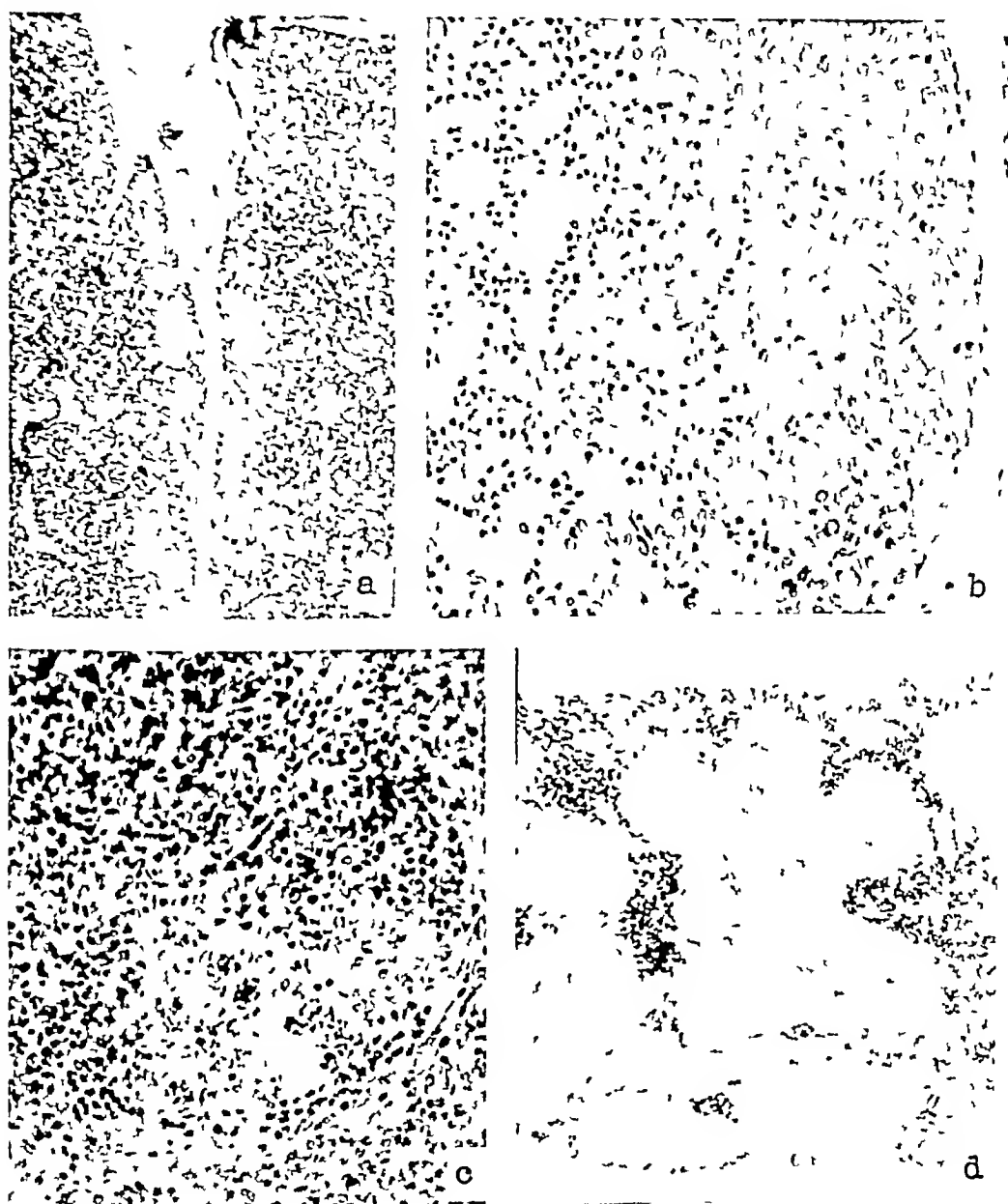


FIG 3

- a Thymus, 2 days after irradiation 5X Narrowed cortical zone, containing few cells Hematoxylin-eosin
- b Thymus, 4 days, 250X Beginning regeneration of cortex Hematoxylin-eosin
- c Spleen, 18 hours after irradiation 250X Partial destruction of follicle, phagocytosis of cellular debris Hematoxylin-eosin
- d Spleen, 48 hours 6X Hemosiderin (black) in red pulp Ferrocy anide-hydrochloric acid-fuchsin

At 4 days a narrow rim of large cells resembling lymphoblasts was visible beneath the capsule (fig 3b)

At 7 days a well developed cortical zone was present, but the medulla still contained fewer lymphocytes than normal. From the 14th day on, the appearance and relative size of both cortex and medulla were within normal limits.

*Spleen.* At 2, 3, 7 and 18 hours nuclear debris was present in large amounts in the Malpighian follicles and to lesser extent in the red pulp. Debris-laden macrophages were clearly seen in some of the follicles (fig. 3c). The small, but fairly numerous centers of erythropoiesis normally present in the mouse spleen, were decreased in size and numbers. Large amounts of brownish, iron-containing pigment appeared in the red pulp. The megakaryocytes were reduced in number and frequently showed pyknotic nuclei. At 24 hours the spleen was greatly reduced in weight. Nuclear debris was seen only in rare macrophages in the follicles which were somewhat reduced in size. Nuclear debris had completely disappeared at 48 hours.



FIG. 4. SPLEEN, 10 DAYS AFTER IRRADIATION. 7X. FOCAL AREA OF HEMATOPOIESIS.  
HEMATOXYLIN-EOSIN

Erythropoietic centers were no longer recognizable. Iron-containing pigment was diffusely distributed through the red pulp, partly phagocytosed in the cells of the condensed reticulum, which often contained numerous red blood cells and few lymphocytes. In Giemsa stained sections the follicles stood out sharply against the uniformly pink-staining red pulp. In sections stained for iron the red pulp took a rather intense blue color, while the follicles took the color of the counter stain only (fig. 3d). Megakaryocytes were markedly reduced in number. The appearance of the spleen remained unchanged at 4 and 7 days, except for an occasional mitosis in the follicles. A marked increase in the size of the spleen occurred between the 7th and 10th day, partly due to increase in the size of the follicles, partly due to formation of subcapsular areas of hemopoiesis (fig. 4). These circumscribed foci of hemopoiesis were frequently visible grossly as light-red or whitish subcapsular

nodules, projecting slightly above the surrounding surface of the spleen. In sections these foci were seen to consist chiefly of round cells with a small rim of cytoplasm with large nuclei and well defined nucleoli (hg. 5). Occasional mitoses were seen. In smears, the majority of these cells were identified as pronormoblasts, among which were a small number of myeloblasts. By the 14th day the spleen had enlarged further and its weight, as well as the size of the Malpighian follicles, was within normal limits. Hemopoiesis was in progress throughout the red pulp. Numerous normoblasts and occasional granulocytes were now intermingled with stem cells. The amount of pigment, already reduced at 10 days, now rarely exceeded that of controls. Megakaryocytes were again present in normal numbers. At 3 and 4 weeks the spleen showed essentially the same appearance as at 2 weeks. Myelopoiesis was somewhat more extensive, but erythropoiesis continued to dominate the picture.

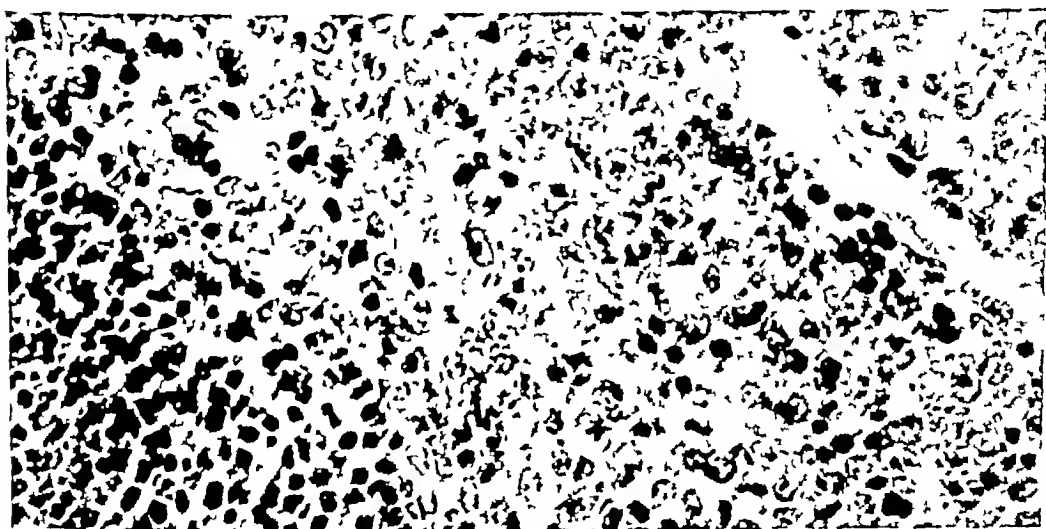


FIG. 5. SPLEEN, 10 DAYS AFTER IRRADIATION. 500X. STEM CELLS IN FOCAL AREA OF HEMATOPOIESIS. EDGE OF MALPIGHIAN FOLLICLE IN LOWER LEFT CORNER.

The accumulation of hemosiderin, noted in the spleen after irradiation, was not found in any other organ examined. Hemosiderin in the spleen was most abundant from the 1st to the 7th day. The period of greatest accumulation of hemosiderin thus coincided with the time of greatest reduction in the size of the spleen. Some of the apparent increase in the amount of pigment may therefore have been due to collapse of the splenic stroma with resulting condensation of pigment.

From the histologic appearance alone it was impossible to decide whether this hemosiderosis developed within 24 hours and remained constant over a period of a week, or whether hemosiderin continued to accumulate during the first week. The hemosiderin present at 24 hours was so abundant that any further increase could hardly have changed the histologic picture.

*Bone marrow.* The diminution and subsequent increase of the total cellular bone marrow are recorded in table 2. Sections showed an increase in the size of blood-filled vascular spaces concomitant with the loss of cellular marrow following irra-

diation A very small amount of cellular debris and slight phagocytosis were noted from 1 to 24 hours after irradiation Our paraffin embedded sections were generally unsuitable for the study of finer cellular details The bone marrow changes, as studied by means of quantitative myelograms, are reported with the hematologic findings

*Summary of the histologic findings* Within 24 hours after irradiation, there was extensive destruction of the cells of the secondary centers and medullary cords of the lymph nodes, almost complete destruction of the thymic cortex, diminution of the lymphocyte content of the thymic medulla and marked decrease in size of Malpighian follicles The weight of the lymphoid tissues was markedly decreased Regeneration of lymphocytes was first noted at 4 days after irradiation The lymphoid tissues regained normal weight at 7-14 days As judged from the histologic appearance, lymphopoiesis was frequently in excess of normal from 14 days on through the rest of the period of observation

The spleen showed marked hemosiderosis from the first to the 7th day Due to the marked collapse of splenic stroma during this time, part of this hemosiderosis was probably more apparent than real No hemosiderosis was found in other organs examined

Extramedullary hemopoiesis was marked in the spleen from the 10th day on and comprised both the myeloid and erythroid series Extramedullary myelopoiesis was also prominent in the lymph nodes at 3 and 4 weeks

#### HEMATOLOGIC FINDINGS

*Cellularity of the bone marrow* decreased during the first 4 days after irradiation The first evidence of recovery was noted at 7 days The total cellularity was still below normal at 2 weeks Over 90 per cent of the available marrow space was occupied by active marrow at 3 and 4 weeks The different cell types showed marked differences in rate of diminution and regeneration

The first regeneration of nucleated red cells of the marrow was noted at 7 days Average indices reached normal at 10 days, but fell again by 14 days and remained below normal throughout the period of observation

The statistically significant fall in nucleated red cells at 3 weeks and the renewed increase at 4 weeks is in essential agreement with the data of Langendorff and Papperitz,<sup>10</sup> who believe that regeneration of hematopoietic tissues after x-ray injury proceeds in waves The possibility of excessive myelopoiesis suppressing erythropoiesis appears to fit the data well, especially as the slight decrease in myelopoiesis at 4 weeks is accompanied by a corresponding increase in nucleated red cells

*The reticulocyte count* in the peripheral blood dropped from its average normal value of 2.0 per cent to 0.2 per cent within 24 hours after irradiation It remained at this low level at 2 and 4 days After 7 days up to 0.8 per cent were found On the 10th day reticulocyte counts varied from 2.0 per cent to 22.0 per cent From the 14th day on reticulocyte levels ranged from 2.0 per cent to 5.0 per cent

The rapid reticulocyte drop within 24 hours after irradiation would indicate a rather short lived reticulocyte stage of the red cell in the mouse

*Total red cell counts*, hematocrit and hemoglobin values dropped only slightly during the first 4 days after irradiation. They were markedly reduced between the 7th and 14th day. The lowest average values were observed in one group of six animals examined and killed on the 10th day (RBC 6,600,000  $\pm$  800,000, Hb 14.0  $\pm$  2.0, hematocrit 33  $\pm$  9). At 3 weeks only slight residual anemia was present. At 4 weeks total red cell counts were frequently within normal limits (Control group RBC 9,600,000  $\pm$  1,300,000, Hb 18.4  $\pm$  1.8, hematocrit 51.0  $\pm$  0.5). In most groups examined the 2-week-values were below the one-week-levels but occasionally the reverse was the case.

*The myelopoietic marrow* diminished more gradually than the red cell precursors. The lowest level was reached at 4 days. Regeneration proceeded quickly and the myelopoietic indices were above normal from the 14th day on. The period of observation is too short to determine whether the slight but significant drop in myelopoiesis at 4 weeks indicates a return to normal values or not.

The peripheral granulocyte counts reflected the marrow changes accurately. As in the marrow the lowest granulocyte level, amounting in some animals to agranulocytosis, was reached at 4 days. There was slight recovery by the 7th day. A marked left shift was present both in the marrow and in the peripheral blood at 10 days.

*Marrow lymphocytes*, normally representing almost 20 per cent of the active marrow cells, dropped to a low value within 24 hours. Lymphocytes in the marrow remained scarce until the end of the observation period, except for an abrupt, temporary rise around the 10th day. The cells recorded as lymphocytes in our 10-day-data included a varying number of large cells with dark blue cytoplasm and with somewhat finer distribution of nuclear chromatin than that of mature lymphocytes. Some of these cells showed great similarity to myeloblasts, but lacked nucleoli. These cells are probably identical with the lymphoidocyte of Pappenheim. We feel that the morphology of these cells is not sufficiently distinctive to permit them to be differentiated from lymphocytes when they are present in small numbers only. We have therefore recorded them with the lymphocytes.

As to the function of these cells, however, there is reason to believe that they are stem-cells of the granulocytic series. Cells of this type did not appear in the peripheral blood, a fact which supports the contention that they are stem cells. Their appearance in the bone marrow coincided with a marked left shift in the granulocytic series and preceded a rapid increase of myelopoiesis. This suggests that these lymphocyte-like cells are stem-cells of the myeloid series.

#### DISCUSSION

There is general agreement in the literature, as reviewed by Dunlap,<sup>11</sup> that lymphoid, myelopoietic and erythropoietic tissues show increasing resistance to x-radiation in the order named. In contrast, the almost complete disappearance of normoblasts from the bone marrow within 24 hours of irradiation in our experiments points to a considerable sensitivity of red cell precursors to radiation. The bone marrow did not show the striking phagocytosis of cellular debris found in the lymphoid tissues. However, in view of the vascularity of the bone marrow and



the probable intravascular development of erythroblastic cells, cellular debris might be expected to be flushed out rapidly. For this reason, the small amount of cellular debris and phagocytosis in the bone marrow does not exclude the possibility of widespread disintegration of red cell precursors, such as would account for the drop in normoblasts and the rapid accumulation of hemosiderin in the spleen. Evidence of direct damage to, and disintegration of, erythroblasts in the bone marrow following x-ray injury has recently been described by Bloom and Bloom.<sup>12</sup> These authors used x-ray dosages similar to ours, but different techniques for the study of the bone marrow. They concluded that the erythroblasts in mice, rabbits and rats are more susceptible to radiation damage than are myelopoietic cells. The erythroblast of the chicken appeared at least as sensitive to radiation as did the small lymphocytes which are commonly found in nodules in the chicken marrow.

Peripheral blood counts showed an almost immediate drop in lymphocytes, and a more slowly developing granulocytopenia. This is in keeping with the longer life span of granulocytes and the slower diminution of myeloid cells in the bone marrow. Granulocyte levels rose again with myeloid regeneration. Lymphopenia was, however, more prolonged than granulocytopenia. At 2 weeks the lymphocyte count was still only 50 per cent of the lower limit of normal, although the lymphoid tissues had regained their normal weight and lymphopoiesis appeared to be slightly in excess of normal. The lymphopenia in the bone marrow was even more marked and prolonged. This lag between resumption of active lymphopoiesis in the tissues and recovery of normal lymphocyte levels in the peripheral blood has been observed in other species.<sup>13</sup> The significance of this lag period is obscure. It is conceivable that the slow return of the peripheral lymphocyte count to normal levels is due to the fact that lymphopoiesis exceeds normal only by a slight margin during regeneration. After replacing the daily loss of lymphocytes from the peripheral blood, only a small excess would remain and a considerable time would be required to restore the peripheral lymphocyte count from its low level following irradiation. On the other hand, the lag in recovery of peripheral lymphocyte counts may be due to disturbances of the mechanism regulating the release of lymphocytes from the tissues and their distribution throughout the body. Most likely several factors are in operation following irradiation and the rate of recovery of the peripheral lymphocyte count should not be expected to reflect necessarily the activity of, or the amount of original damage to, the lymphoid tissues.

No evidence of diminution or proliferation of the reticuloendothelial cells was found. The only function of the cells of the RES that can be studied morphologically, their ability to phagocytose, appeared to be intact throughout the period of observation. In the lymph nodes phagocytosis of cellular debris was extensive during the first 24 hours, due to extensive destruction of lymphocytes, and was again marked, in conjunction with active regeneration of lymphocytes, from the 4th day on. In the spleen large amounts of hemosiderin were present in phagocytic cells from the 1st to the 7th day after irradiation. The ability of the RES to phagocytose appeared also intact in experiments (unpublished) in which carbon particles were injected intravenously in mice of the same strain, age and sex, at varying intervals after irradiation with 400 r. In these experiments active phagocytosis of

carbon particles by the reticuloendothelial cells of the liver, spleen and bone marrow was comparable to, or in excess of, that observed in nonirradiated mice

Our basic problem, to estimate the duration of suppression of lymphoid and hematopoietic activity, can receive only a qualified answer. Estimates based on the histologic appearance of the lymphoid tissue suggest that the limit of marked suppression of lymphopoiesis was about 14 days. Peripheral lymphocyte levels were still below normal at that time and this would indicate a longer time interval between irradiation and recovery. Total granulocyte counts reached normal levels at 14 days. Again estimates of the recovery time may vary somewhat, as the number of fully segmented granulocytes reached normal levels somewhat later than the total granulocyte count. The practical problem is simplified by the fact that damage to the myeloid tissues, though probably of slighter degree, is still quite severe during the first 10 days. This would certainly leave too narrow a time margin for any experiments designed to demonstrate the relative function of granulocytes and lymphocytes based upon a supposed selective suppression of one cell type. The lack of any morphologic evidence of damage to the reticuloendothelial system suggests that functional studies concerned with the relative functions of the lymphoid tissues and the RES may be undertaken during the first 2 weeks following irradiation under the experimental conditions used. Such experiments must be interpreted cautiously because of the concomitant damage to the bone marrow and because of the possibility that the RES may have been altered in functional capacities other than its ability to phagocytose.

#### SUMMARY

Quantitative myelograms, peripheral blood counts and the histologic appearance of lymphoid tissues were studied in albino mice of the CFW strain during a period of 4 weeks following single whole body radiation of medium dosage.

Nucleated red blood cells disappeared almost completely from the marrow within 24 hours after irradiation. Regeneration of the erythroid series commenced around the 7th day. Only mild to moderate anemia developed, presumably due to the longevity of red blood cells.

Suppression of mitotic activity and diminution of the myeloid marrow was marked during the first week after irradiation. Granulocytopenia in the peripheral blood was severe for a very short period only. In contrast, early damage to the lymphoid tissue was more pronounced and lymphopenia was of longer duration than granulocytopenia in spite of early regeneration of the lymphoid tissues. Differences in the relative amount of damage to lymphoid and myeloid tissues and in their rates of regeneration were not of sufficient magnitude to indicate a truly selective damage to the lymphoid tissues under the conditions of our experiments.

No morphologic evidence of damage to the reticuloendothelial system was found. The possible use of irradiated animals for functional studies of the lymphoid apparatus is discussed.

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## PLATELET ADHESIVENESS

Bj MURRAY WEINER, M D , KURT ZELTMACHER, M D , CARL REICH, M D ,  
AND SHEPARD SHAPIRO, M D

IN 1941, Helen Wright<sup>1</sup> described a method for estimating the adhesive quality of platelets. Confirmation of the original findings by Spooner and Meyer<sup>2</sup> established the procedure as a means of distinguishing this property of platelets. It is the purpose of the present report to evaluate the usefulness of the method in clinical medicine.

### METHOD

Five ml of blood is obtained by clean venipuncture with a dry needle and syringe, and delivered into a small dry bottle containing 8 mg potassium oxalate and 4 mg ammonium oxalate. \* The bottle is gently rotated to aid solution of the oxalate. Within 10 minutes a 2 ml sample of this oxalated blood is pipetted into a glass tube with a bulbous dilatation at one end. The tube is then rotated at 7 r p m. Samples for serial platelet counts are withdrawn before rotation is begun, and every 20 minutes for 80 minutes. The platelet counts are made using the standard red cell pipet with Reese-Ecker diluting fluid to which 2 per cent formaldehyde has been added. The diluting fluid is autoclaved after it is prepared, and stored at refrigerator temperature. Each portion is filtered before it is used. The counting chambers are placed on wet filter paper and covered with a petri dish after being loaded. This permits the platelets to settle without drying on the chamber for about a half hour before the count is made. By this technic large clumps of platelets are rarely seen, and microorganisms which may be confused with platelets are kept at a minimum.

The serial platelet counts are calculated in terms of per cent of the initial count, and a curve is plotted with the number of remaining platelets as ordinate and the time in the rotating tube as abscissae. The curve then represents the proportion of platelets which have failed to adhere to the wall of the rotating tube. In normal individuals, platelet counts usually fall to about 30 per cent of the initial count in 80 minutes. Adhesiveness determinations carried out in 22 adults without evidence of disturbed coagulation or phenomena indicative of vascular alteration, yielded counts of from 25 per cent to 40 per cent of the initial count in 80 minutes. In almost every case, the estimations were made in duplicate. The initial counts ranged from 140,000 to 220,000 per cmm. The data obtained in the 22 subjects is summarized in the solid curves of figure 3.

Since platelet counts vary widely when made by different observers, the counts have been made by one person especially practiced in the procedure, who had attained thoroughly consistent manipulative characteristics. We have found the procedure time consuming and inconsistent when the technic was varied only slightly. Prothrombin estimations were made by a method previously described.<sup>3</sup> Fibrinogen levels were established by determination of the protein content of plasma before and after the contained fibrinogen was coagulated and removed.

### RESULTS

The present report embraces a study of the curves of platelet adhesiveness in 110 instances which include both normal and a large variety of disease states.

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\* Since this paper has been submitted, we have been using 0.2 mg heparin per ml blood in place of the oxalate.

No abnormality in platelet adhesiveness was demonstrated in a large variety of diseases in which disturbed coagulation was not a factor. These include infectious mononucleosis, 2 cases, erythema multiforme, 1 case, pulmonary hemorrhage secondary to bronchiectasis, 1 case, duodenal ulcer, 2 cases, one actively bleeding, rheumatic heart disease, 3 cases, arteriosclerotic heart disease, 4 cases, hypertensive heart disease, 4 cases, diabetes mellitus, 2 cases, rheumatoid arthritis, 2 cases, amyloid disease, 1 case, subarachnoid hemorrhage, 1 case, and multiple sclerosis, 1 case. The detailed data which follow represent instances in which altered adhesiveness of the platelets and/or coagulability were demonstrated.

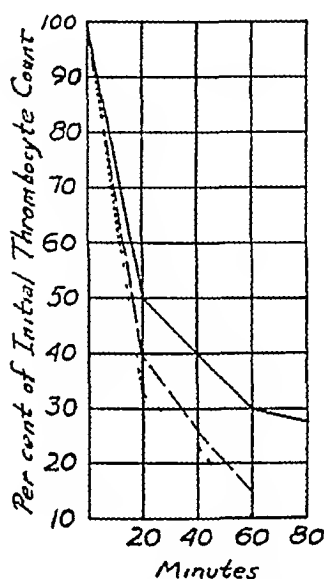


FIG. 1. PATIENT G. EFFECT OF HYPERPROTHROMBINEMIA INDUCED BY LARGE DOSES OF VITAMIN K.

Solid line indicates Control (diluted, 12.5 per cent, plasma prothrombin Time, 42 sec normal). Broken line indicates third day (12.5 per cent plasma prothrombin time, 35.2 sec). Dotted line indicates fourth day (12.5 per cent plasma prothrombin time, 31.8 sec).

### 1. Platelet Adhesiveness in Hypercoagulable Blood

The adhesiveness of the platelets was studied in conditions in which the blood was hypercoagulable. It is possible to induce this condition experimentally in some subjects with normal liver function.<sup>4</sup> It occurs spontaneously in some cases of intravascular thrombosis. It is detectable by a reduction in prothrombin time to a level below the limits of normal (hyperprothrombinemia) and/or thrombocytosis.<sup>5</sup>

#### (a) Hyperprothrombinemia Induced by Vitamin K<sup>4</sup>

Synthetic vitamin K (Synkayvite) (Tetrasodium 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester) 76 mg daily on four successive days was given to a subject with normal liver function, in order to induce hyperprothrombinemia (fig. 1).

Day of therapy	Prothrombin time of diluted (12.5%) plasma*	Fibrinogen	Initial platelets in 80 minutes
	<i>seconds</i>	<i>mgm/100 ml</i>	<i>%</i>
Control	42.2	410	29
Third	25.2	400	13
Fourth	31.8	450	9
Sixth	42.0		20

\* Normal range 37-42 seconds

(b) *Reactive Thrombocytosis and Hyperprothrombinemia in Thrombosis*<sup>5</sup>

Diagnosis	Prothrombin time of diluted (12.5%) plasma*	Platelet count per cmm	Initial platelets in 80 minutes
	<i>seconds</i>		<i>%</i>
Acute venous thrombosis	33	600,000	30
Acute coronary thrombosis	Not made	600,000	24

\* Normal range 37-42 seconds

2 *Platelet Adhesiveness in Hypocoagulable Blood*

(a) *Dicumarol-induced Hypoprothrombinemia*<sup>2, 6</sup> (fig. 2)

Prothrombin time of whole plasma	Initial platelets in eighty minutes	Diagnosis and remarks
<i>seconds</i>	<i>%</i>	
24.0	53	Pulmonary embolus, on dicumarol therapy
30.0	53	
24.0	55	
15.0	30	Recovered Therapy withdrawn

(b) *Thrombocytopenic Purpura*

A 20 year old white male showed the clinical manifestations of thrombocytopenic purpura. The platelet counts varied between 22,000 and 220,000 and were usually below 80,000 per cmm. The intensity of the clinical symptoms could not be correlated with the degree of thrombocytopenia. The prothrombin time, fibrinogen concentration, clot retraction and clotting time were consistently normal. Four determinations of platelet adhesiveness were made. The initial counts varied between 60,000 and 220,000. In none of these did the count fall below 47 per cent of the initial value after eighty minutes. The average count after eighty minutes was 53 per cent. The average of the four determinations is compared to the normal in figure 3.

(c) *Spontaneous Purpura in Pregnancy*

A 26 year old female para 1 gravida 1, commenced to show diffuse purpuric hemorrhages during the sixth month of pregnancy. Physical examination revealed no other positive findings. Prothrombin time, fibrinogen concentration and clotting

# PLATELET ADHESIVENESS

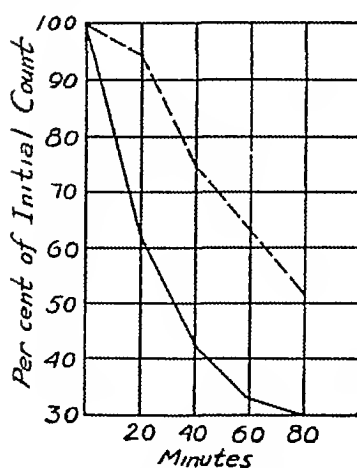


FIG 2 PATIENT W

*Solid line indicates control Broken line indicates after dicumarol (prothrombinopenia)*

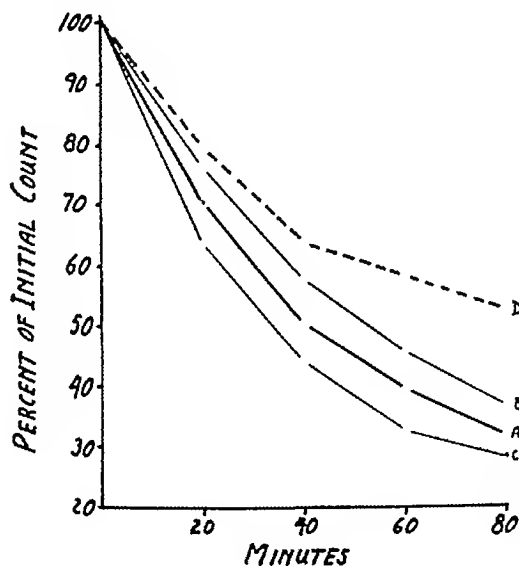


FIG 3 PLATELET ADHESIVENESS IN THE NORMAL AND IN THROMBOCYTOPENIC PURPURA

Curve A represents the average value of 22 normal individuals. Curves B and C represent the average deviation of these normals. Curve D is the average of 4 estimations made in a patient with thrombocytopenic purpura. The initial platelet counts varied between 60,000 and 220,000.

time were normal. The platelet count was 158,000 and the adhesiveness curve yielded a fall to 67 per cent of the initial count in 80 minutes. The purpuric condition subsided spontaneously one month later, and the adhesiveness curve became normal.

minutes	During hemorrhagic phase	After recovery
	per cent of initial count	%
20	84	75
40	85	48
60	80	42
80	67	32

(d) *Prolonged Clotting Time and Hemorrhage*

A male adult with chronic rheumatic valvular disease, exhibited continuous oozing of blood from the gums for three days after a tooth was extracted. The bleeding and prothrombin times were normal, but the clotting time was slightly prolonged to seven and one-half minutes by the rotating tube method<sup>2</sup> (Upper limit of normal is six and one-half minutes). At that time the platelet count was 140,000 and the adhesiveness curve fell to 30 per cent in 80 minutes. After the hemorrhage ceased, the clotting time became reduced to normal (four and one-half minutes) and the platelet count increased to 370,000 and the adhesiveness curve yielded a reduction to 18 per cent of the initial count in sixty minutes, which is indicative of an increase beyond normal.

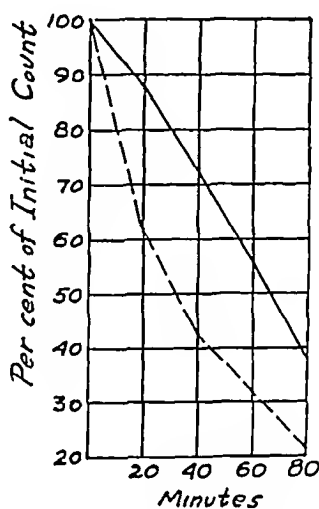


FIG. 4. PATIENT R. HEMOPHILIA

Solid line indicates control. Broken line indicates coagulation time reduced by Fraction 1.

(e) *Chronic Myelogenous Leukemia*

A male adult with chronic myelogenous leukemia began to show diffuse purpura concomitantly with marked increase in peripheral myeloblasts. The platelet count was 262,000 but the adhesiveness curve fell only to 82 per cent of the initial count in eighty minutes. The clotting time and prothrombin time were normal. The patient died of exsanguination three days later.

(f) *Hemophilia: Effect of Antihemophilic Fraction one\**

An 18 year old Negro male of unknown parentage showed the coagulation defects characteristic of hemophilia since early childhood. The bleeding time, prothrombin time, and fibrinogen content of the plasma were normal. The clotting time, when the present study was made, was sixty-nine minutes by the rotating tube method (Normal = four to six and one-half minutes). The platelet adhesive-

\* Fraction 1 was prepared from blood collected by the American Red Cross under a contract with the Office of Scientific Research and Development and Harvard University. It was supplied at the courtesy of Dr. Louis K. Diamond.



ness curve fell to 38 per cent in eighty minutes (upper limit of normal) Anti-hemophiliac fraction 1 was administered intravenously. The coagulation time became reduced to 16 minutes and the adhesiveness of the platelets increased to 23 per cent in eighty minutes (fig. 4).

A 40 year old white male with hemophilia the diagnosis was established in early childhood. The coagulation time ranged between 37 and 47 minutes by the rotating tube method (normal = four to six and one-half minutes). On numerous occasions, platelet adhesiveness was determined and averaged 32.5 per cent of the initial count in eighty minutes. He was given fraction 1 intravenously, and the clotting time was reduced to between four and ten minutes at various times. Platelet adhesiveness estimated when clotting time was reduced to normal, yielded an average of 24 per cent (two estimations) in eighty minutes.

#### SUMMARY OF FINDINGS

In the presence of hypercoagulable blood occurring spontaneously in association with thrombosis or artificially induced by large doses of vitamin K, the adhesiveness of the platelets was generally increased. In the spontaneously occurring cases, the change was not constant, instances of normal adhesiveness having been observed even in the presence of marked thrombocytosis. Hypocoagulable blood, whether occurring as part of a blood dyscrasia, or induced by dicumarol, was accompanied more consistently by decreased adhesiveness of the platelets. In hemophilia, the clotting time and the adhesiveness simultaneously were reduced following restoration of coagulability to normal by Fraction 1.

In several cases exhibiting hemorrhagic phenomena, decreased stickiness of the platelets was the only defect found in the coagulation mechanism.

#### DISCUSSION

Adhesiveness of platelets appears to be dependent upon at least two factors: the intrinsic properties of the thrombocyte surface, and the character of the medium in which the platelets are suspended. It is believed<sup>9</sup> that fibrinogen is converted into fibrin on the surface of the platelets, and that adhesiveness is the result of this change. It would follow then, that inhibition of the clotting mechanism should impair the adhesive capacity of the thrombocytes and conversely, that augmented coagulability should accelerate adhesiveness. The findings confirm the first hypothesis but support the latter with less constancy in hypercoagulability accompanying thrombosis. Hypocoagulable blood may be induced by deficiency of any component factor of the clotting mechanism. Thrombosis, on the other hand, appears to be the result of a combination of events of which hypercoagulability of the blood may, or may not, be one. In addition, in thrombotic disorders, anticoagulants may be elaborated into the blood stream, presumably as a protective mechanism, so that, when examined *in vitro*, the behavior of the thrombocytes may vary according to the particular type of response operating at the time the specimen is obtained. Nevertheless it seems logical to assume that in a given case, the detection of increased adhesiveness of the thrombocytes warrants pursuit of the possibility of thrombosis being present or imminent. In earlier studies<sup>2</sup> we have found evidence of increased prothrombin activity in the presence of intravascular

thrombosis, and have suggested it as a diagnostic aid, both in the detection of thrombosis (especially in inaccessible locations) as well as in the selection of probable candidates for the complications. The practical application of the detection of hypercoagulability of the blood, particularly in postoperative cases, and following parturition, is obvious. Studies of this kind in inadequately understood disease states accompanied by thrombosis such as thrombocytopenia with platelet thrombus formation<sup>10</sup> might shed some light on the mechanism underlying the disorder.

The correlation noted in some of our cases, between the state of coagulability of the blood and adhesiveness of the platelets, seems to support the fibrin theory of stickiness of thrombocytes. A few observations seem to require additional explanations. Microstaining studies have not revealed fibrin on platelet surfaces.<sup>11</sup> In the presence of the markedly increased clotting time in hemophilia, the detectable differences in adhesiveness as compared with the normal have been relatively slight, while in dicumarol-induced prothrombinopenia of moderate severity, much greater interference in platelet adhesiveness is the rule. In the cases in which fibrinogen determinations were made, the changes in platelet adhesiveness were found to occur independently of the concentration of this protein in the plasma.

The deficiency in adhesiveness of the platelets noted in hemophilia is of particular interest. Since the blood is hypocoagulable, it is not possible to determine from the present study whether or not the platelets are functionally defective in hemophilia. The stickiness of the thrombocytes is promptly increased when the coagulation defect is corrected by Fraction 1. It is possible that the improved adhesiveness is an expression of the more normal coagulability of the blood.<sup>12</sup> In thrombocytopenic purpura, we have found Fraction 1 to be without effect.<sup>13</sup> This phase of the study is being extended in this laboratory.

The two cases of bleeding described above (cases c and e) in which deficient adhesiveness of platelets was the only detectable defect in the coagulation mechanism, suggest that a knowledge of the state of the stickiness of thrombocytes may be of assistance in explaining instances of hemorrhagic tendencies which do not fall in categorized syndromes.

#### SUMMARY AND CONCLUSIONS

The method described by Helen Wright for estimation of the adhesiveness of platelets has been found to be reproducible. Adhesiveness of platelets is reduced in the presence of hypocoagulable blood. It may or may not be enhanced when the blood is hypercoagulable. In hemophilia, the adhesiveness of platelets has been found to become increased beyond the premedication level when the coagulation defect is corrected by Fraction 1 derived from human plasma. Two cases exhibiting bleeding are described in which deficient adhesiveness of the thrombocytes was the only demonstrable defect noted in the coagulation mechanism.

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## CHRONIC LEG ULCER IN DISEASES OF THE BLOOD

By BENJAMIN R. GENDEL, M.D.

CHRONIC LEG ULCERS have been described as a complication of several diseases of the blood and are best known in association with sickle cell anemia. Diggs and Ching<sup>1</sup> reported that chronic ulcers or scars of old ulcers were present in 75 per cent of adults with this disease. However, it is less commonly appreciated that other diseases of the blood may present the complication of chronic leg ulcer. In 1925, Gansslen<sup>2</sup> mentioned a 19 year old patient with congenital (hereditary) hemolytic anemia who, over a period of nine months, had recurrent leg ulcers which resisted therapy, but healed eight days after splenectomy. Since that time over twenty patients with hemolytic anemia have been reported with chronic leg ulcers. The exact incidence of this complication is difficult to determine, but Vaughan<sup>3</sup> described the occurrence of leg ulcers in three patients of a series of 18 with congenital hemolytic anemia. Taylor<sup>4</sup> reported one patient with leg ulcer among 43 patients with hemolytic anemia. The combined incidence for the complication of leg ulcer in these two series is 6.5 per cent. Following the report of Taylor which summarized the previous literature, two cases were reported by Leger and Orr<sup>5</sup> and another by McGovern.<sup>6</sup> The ulcer complicating hemolytic anemia occurs in younger people and is located on the lower one-third of the legs. Ulcers were usually several centimeters in diameter and were surrounded by an area of pigmented skin. In half the cases, the ulcers were bilateral. In the majority of instances healing occurred rapidly after splenectomy, although the lesions proved refractory to previous therapeutic efforts.

Several French authors<sup>7-8</sup> have noted the association of chronic leg ulcer with splenomegaly. The diagnosis in their cases is not clear, but they seem to represent patients with Banti's syndrome of congestive splenomegaly because leukopenia and recurrent hematemesis were present. Witts<sup>9</sup> reported two patients with idiopathic thrombocytopenic purpura complicated by indolent leg ulcers. His first patient was a 21 year old female with thrombocytopenic purpura of nine years' duration. Six years earlier an ulcer appeared on the left leg which did not heal for two years. Subsequently an ulcer appeared on the right leg, but healed in less time. Both remained healed thereafter. The second patient, a 26 year old man with a lifelong history of excessive bruising and bleeding, had chronic leg ulcer for eight or ten years. The weight of the spleen removed at operation was 122 Gm. In this case there was no improvement of either the general condition or the leg ulcer after splenectomy. Witt also stated that leg ulcers occurred in Gaucher's disease.

The unique occurrence of leg ulcer in pernicious anemia was reported by Lasch.<sup>10</sup>

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His patient had bilateral, symmetrical, deep ulcers above the ankles almost as large as the palm of one's hand. Varicose veins were not present. The patient had the typical picture of pernicious anemia with slight jaundice, histamine fast achylia, glossitis, macrocytic anemia with high color index, and megaloblastic bone marrow. The spleen was slightly enlarged. Following treatment with liver extract, healing began coincident with the reticulocyte peak on the fifth day, and the ulcer healed completely. A patient with chronic hemolytic polycythemia was described by Rau and co-workers.<sup>11</sup> Their patient, a 37 year old female with a five year history of fatigue, had ulcers of the lower legs. Blood studies revealed a polycythemia, spherocytosis and increased fragility of the red cells.

It is apparent from the preceding review of the literature that leg ulceration may occur in the course of a number of diseases associated with hemolytic anemia or splenomegaly. It is the purpose of this report to describe two patients with disorders of the blood aside from sickle cell anemia, who had complicating leg ulcer, and to discuss some of the possible mechanisms for this interesting association. In one of these patients the leg ulcer was the first symptom of the disease.

### CASE REPORTS

*Case 1* The patient, a 52 year old white man, entered the hospital because of recurrent right upper quadrant pain and jaundice, of seven years duration. These attacks were intermittent and accompanied by dark urine, light stools, fever and vomiting. In May and October, 1946, the patient had similar attacks of obstructive jaundice. Splenomegaly was noted several years before admission. The patient also complained of a chronic leg ulcer just above the left ankle of about twenty-seven years duration. This was previously considered to be a varicose ulcer despite the absence of varicose veins. A skin graft had been done in another hospital in November, 1946, prior to his admission to this hospital, and healing had progressed satisfactorily, although the healing ulcer exuded a serous fluid on dependency of the leg.

The past history revealed that since 1932 the patient had recurrent pain in both knees, occasionally accompanied by slight swelling. An uneventful appendectomy had been performed in 1932. In October, 1945, the patient was hospitalized at another hospital for an acute nephritis characterized by edema, hypertension, albuminuria, and hematuria, all of which gradually cleared up in several months.

The family history revealed no instance of a similar disease. A complete examination of all members of the family was not possible, but two siblings who were examined showed no evidence of hemolytic anemia.

Physical examination revealed an acutely ill, febrile, anemic man with moderate icterus. The blood pressure was 140/80. Heart and lungs were normal. No significant lymph node enlargement was present. The liver was not palpable but there was tenderness over the gallbladder region. Spleen was readily palpable three fingerbreadths below the left costal margin. There was a well healed McBurney scar. An oval ulcer covered by a recent skin graft was noted just above the external malleolus of the left leg (fig. 1). It measured 2.6 cm. in its longest diameter. The remainder of the examination was not significant.

The admission blood count revealed a red cell count of 2,200,000, hemoglobin, 11.5 Gm., white cells, 9,500, 73 per cent polys, 24 per cent lymphocytes, 1 per cent monocytes, and 2 per cent eosinophils. Reticulocytes 8.4 per cent. Platelets normal. The subsequent blood counts are summarized in table 1. The serum bilirubin on admission was 1.3 mgm. Kahn was negative. Repeated urine examinations revealed a low, fixed specific gravity but were negative for albumin and sugar and no abnormal elements were found in the sediment. Repeated urea clearance tests varied between 37 per cent and 59 per cent of normal. Examination of a wet preparation of the peripheral blood revealed definite spherocytes (fig. 2). Increased fragility of the red cells in hypotonic saline was noted. Cephalin flocculation, 4 plus in 48 hours. Alkaline phosphatase, 2.6 King-Armstrong units. Prothrombin time, 88 per cent of normal. X-ray examination of the chest and both knees revealed no significant pathology.

After admission, fever continued for the first five days of hospitalization. Tests of the blood for hemo-



FIG 1a WET PREPARATION SHOWING SPHEROCYTES CASE I



FIG 1b A SIMILAR PREPARATION SHOWING NORMAL BLOOD FOR COMPARISON WITH FIG 1a

TABLE I

Date	Red blood cells	Hemo globin	Hema tocrit vol	White blood cells	Differential	Ret count	Platelet
	millions	gms/100 cc	%	thousands		%	thousands
1-10-47	2 22	11 5	31	10 35	P75 L24 E1	8 4	195
1-17	3 75	13 2	31	9 15	P66 L32 M1 E1	5 4	365
1-24	3 02	13 6		8 9	P65 L34 E1	1 2	
1-31	3 56	13 2		13 5	P78 L17 M3 E2	2 9	
Splenectomy 2-3-47							
2-3	3 5	12 8		28 05	P96 L2 E2	4 2	
2-5	3 75	12 8		16 4	P71 L16 M10 E3		1,660
2-28	4 10	14 4	38	14 75	P63 L30 E5 B2		
3-10	4 70	14 4		12 1	P76 L22 E2	0 8	836
4-10	5 3	15 5		13 8	P63 L34 M1 E2		
5-2	5 6	16 2		18 8	P52 L39 M3 E6		
Cholecystectomy 5-6-47							
5-12	4 3	13 4	39	30 75	P82 L17 1 Myelocyte		430
6-6	4 6	14 0	39	11 5	P45 L51 E4		600
7-9	4 8	14 4		18 5	P58 L28 M14 E1		450
Hemorrhoidectomy 7-29-47							
11-10	5 8	16 4		17 4	P62 L33 E5	0 2	672



FIG 2 CASE 1 LEG ULCER AFTER SKIN GRAFTING

lysins and agglutinins, using both saline and albumin as diluents, revealed no evidence of abnormal agglutinins or hemolysins. A splenectomy was performed on Feb 3, 1947, and a large spleen weighing 1,120 Gm was removed. Three small pieces of tissue considered to be accessory spleens were also removed. On histologic examination the latter were found to be hyperplastic lymph nodes. Following splenectomy, the patient's general condition improved remarkably and the blood count returned to normal. Spherocytes persisted, although in lesser numbers than prior to splenectomy. The ulcer healed completely and has remained well-healed ever since. On May 6, 1947, a cholecystectomy was performed. The gallbladder revealed evidences of chronic cholecystitis and contained several gallstones. A hemorrhoidectomy was performed on July 29, 1947, because of severe symptomatic hemorrhoids. The blood picture has been normal since splenectomy except for a slight persistent spherocytosis, slight leukocytosis and thrombocytosis.

*Case 2* \* A 60 year old white man gave a history of illness since the autumn of 1943 when he was admitted to a hospital in coma with slight neck rigidity and paresis of the right external rectus muscle. There was a bloody ooze from the gums. The spleen was found to be enlarged. Spinal fluid examination revealed a xanthochromic fluid under increased pressure. Blood studies revealed a pancytopenia. A diagnosis was made of subarachnoid hemorrhage due to an underlying blood dyscrasia of unknown type. Treatment consisted of general supportive therapy including blood transfusions, as a result of which the

TABLE 2

Date	Red blood cells	Hemoglobin	White blood count	Platelet count	Differential
	<i>millions</i>	<i>gm/100 cc</i>	<i>thousands</i>		
Oct 8, 1943	2.91	10.0	3.05	Markedly decreased	P62 L23 M1 E6 Myelocytes 8
Oct 12, 1943	3.04	10.5	2.75	94,800	
Oct 15, 1943	3.58	12.5	4.2		P64 L19 E10 Myelocytes 7
Dec 23, 1946	3.62	11.5	4.25	80,000	P71 L25 M1 E3
Dec 26, 1946	3.27	11.4	3.35	63,000	P54 L3 M3 E3
Dec 27, 1946	4.35			62,000	
Dec 30, 1946	4.41		4.9	56,000	
Mar 14, 1947	2.91		7.05		P82 L4 M11 Myelocytes 3

patient gradually regained consciousness and the bleeding from the gums ceased. Further workup did not elucidate the nature of the dyscrasia and the patient returned to his home several hundred miles away. Although he was instructed to return for study on completion of his convalescence at home, he was not observed again until Dec 1946 when he consulted a urologist because of bloody urine beginning in Nov 1946 and persisting for four to six weeks. Studies revealed no local cause for the bleeding. A pancytopenia was again noted.

The past history revealed that seven years previously the patient had a tooth extracted and following this there was severe bleeding which necessitated blood transfusion.

The patient's father was reported to have had hemorrhage from the lung presumably due to an infection alleged not to be tuberculosis. A sister had an episode of severe bleeding following extraction of teeth.

The physical examination revealed a markedly obese, elderly, white male. Blood pressure 120/80. Heart and lungs were normal to physical examination. No lymph node enlargement was found. The abdomen was extremely difficult to palpate due to the extreme obesity but the spleen could be felt three fingers below the costal margin. The liver appeared to be enlarged two fingers below the costal margin to percussion. There was a superficial ulcer, about 4 x 6 cm, on the anterior surface of the right leg just above the malleoli. No varicosities were noted.

\* The author thanks Dr. Lytle Motley of Memphis, Tennessee, for the opportunity to see this patient and for his permission to report this case.



The blood counts are summarized in table 2. A sternal puncture revealed a nonspecific hyperplasia of the marrow.

The patient was treated with repeated transfusions and showed a slight improvement. He returned home again only to return on March 14, 1947, in a comatose condition. A history of head injury two weeks prior to admission was obtained, followed in ten days by drowsiness, progressing to coma two days before admission. Lumbar puncture revealed a bloody fluid which became clear toward the end of the tap. The patient was treated with nasal oxygen, penicillin and other supportive measures. His temperature rose progressively to 106.6 F. on the third hospital day when he expired on March 16, 1947.

An autopsy was performed by Dr. A. Golden, Baptist Memorial Hospital, Memphis, Tennessee, on the same day. The salient features included the presence of the previously noted leg ulcer on the lower right leg measuring approximately 10 cm. in length and 4 cm. in width. No evidence of intracranial hemorrhage was noted. Numerous pin-point hemorrhages were noted in the intestinal tract and the stomach and intestines were filled with a hemorrhagic fluid. Esophageal varices were noted. The liver (1220 Gm.) revealed typical hobnail cirrhosis (Laennec's) and the spleen (800 Gm.) showed congestive changes. The final anatomical diagnosis was: Cirrhosis of liver with portal obstruction, splenomegaly, portal hypertensive type, esophageal varices, and hyperplasia of the bone marrow.

### DISCUSSION

The first patient presented a typical hemolytic anemia, the earliest symptom of which was the leg ulcer. This proved resistant to treatment over a period of twenty-seven years and finally healed after skin-grafting and splenectomy. Based on previous reports it is possible that splenectomy alone would have been equally efficacious. It is interesting that the fundamental condition was not appreciated and the diagnosis during this time was varicose ulcer, despite the absence of varicose veins. It was impossible to determine definitely whether or not the patient had an hereditary type of hemolytic anemia, because of inability to examine all of the other members of the family, but the persistence of spherocytes after splenectomy suggested the hereditary variety. The second patient presented a difficult problem in diagnosis. At different times a variety of diagnoses were considered but the autopsy revealed cirrhosis of the liver and congestive splenomegaly (Banti's syndrome). The pancytopenia could then be explained by assuming a secondary hypersplenism which developed as a result of the congestive splenomegaly. The patient presented the unusual complication of leg ulcer in association with Banti's syndrome. Similar cases were reported previously by Lombard and Nanta<sup>7</sup> and by Gregoire and Weill.<sup>8</sup>

The pathology of the ulcerated lesions of the legs has not been characteristic. Essentially, the ulcers revealed a nonspecific infiltration with inflammatory cells, sometimes associated with fibrosis and hemorrhage.<sup>3, 6, 7</sup> The main value of biopsy would be to rule out other specific leg ulcers such as those due to syphilis or tuberculosis. Biopsies were not undertaken in either of these patients.

The pathogenesis of the leg ulcers accompanying these varied disorders of the blood has not been satisfactorily explained. In the case of sickle cell anemia, it is supposed that the general tendency to vascular thromboses might result in local thrombosis of the skin of the legs and the development of leg ulcer. However, this explanation would not satisfactorily account for the ulcers occurring in the other diseases, in which the tendency to thrombosis has not been noted. Hemolysis occurs in most of these diseases but is not characteristic of either thrombocytopenic purpura or Banti's syndrome. The common denominator would seem to be related

to the spleen. All the diseases in which leg ulcers have been noted, have been accompanied by splenomegaly, with the exception of thrombocytopenic purpura and some patients with sickle cell anemia in whom the spleen may be atrophic. In the former, evidence has been presented for a splenic inhibition of the bone marrow (hypersplenism) to account for the deficiency of platelets.<sup>12</sup> Sickle cell anemia is a hemolytic anemia, and on this basis it can be assumed that there may be some excessive splenic activity accompanying the increased activity of the spleen due to sequestration of the abnormal red cells.

The rapid healing of the ulcer frequently noted following splenectomy suggests a causal relationship between the two, rather than a coincidental occurrence. It is noteworthy that prior to splenectomy many forms of treatment including bed rest have failed to produce healing. Consequently it is suggested that the leg ulcers are another manifestation of a remote effect of the spleen, perhaps a form of hypersplenism mediated in a manner not yet known.

#### SUMMARY

The literature pertaining to the association of chronic leg ulcer with diseases of the blood other than sickle cell anemia has been reviewed.

Two patients with this association have been presented. One patient had a hemolytic anemia and the other a pancytopenia (secondary hypersplenism) in association with congestive splenomegaly due to cirrhosis of the liver.

It is suggested that the association of leg ulcer with these various diseases of the blood is related in an unknown manner to either splenomegaly or hyperfunction of the spleen (hypersplenism).

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## A QUANTITATIVE METHOD FOR THE DETERMINATION AND CHARTING OF THE ERYTHROCYTE HYPOTONIC FRAGILITY

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THE DETERMINATION of the degree of hemolysis of the red blood cells in hypotonic salt solutions (hypotonic fragility<sup>1</sup>) has continued to be of considerable interest since Duncan Johann,<sup>1</sup> in 1867, and Malassez,<sup>2, 3, 4</sup> in 1873, instituted the method of using salt solutions to detect the varying resistance of red cells to hemolysis. Since then, many new technics have been described and much controversial data regarding hypotonic fragility and its interpretation have been amassed. It is only in recent years that full recognition of the importance of this test as a measurement of red cell thickness has been realized.

It is the purpose of this paper to present a new technic for the measurement of hypotonic fragility, based first on the photoelectric determination of the degree of hemolysis and second, upon calculation of the differences in the degree of hemolysis occurring in successive dilutions of hypotonic salt solution. The graphic depiction of these differences, or hemolytic increments, has proved of great value in assessing the red cell population according to its thickness variation, (thickness in relation to diameter), thus indirectly allowing evaluation of the probable type of hemolytic process present.

### I REVIEW OF LITERATURE

So many methods for determining the hypotonic fragility have been described that a thorough review of the literature is extremely difficult. In 1867, Duncan Johann<sup>1</sup> reported an increased fragility in salt solutions of the red blood cells of patients with chlorosis. He did not elaborate on his observations and apparently made no attempts to standardize his method.

Malassez,<sup>2, 3, 4</sup> while attempting to devise a practical method for the counting of red blood cells, observed that cells from different individuals become hemolyzed at different time intervals in the diluting fluid used (a mixture of gum arabic with equal parts of solutions of sodium sulfate and sodium chloride). Malassez then described a quantitative method for the determination of red cell hemolysis consisting of the use of the "Potain mixer," (the original red blood cell counting pipet) and in the performance of red cell counts at different time intervals, thus determining the number of residual nonhemolyzed red cells. The time element was of paramount importance. Urcelay,<sup>5</sup> Malassez's pupil, used a sodium sulfate solution with a specific gravity of 1.010. He established a curve based on the number of nonhemolyzed erythrocytes at different time intervals and concluded that in

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normal blood two groups of red cells were present a smaller one of easily hemolyzed red cells and a larger one of cells which were more resistant to hemolysis

Chanel<sup>6</sup> used three different dilutions of sodium sulfate in water, substituting the factor of dilution for the time factor and performing red blood cell counts to determine the number of nonhemolyzed red blood cells Simmel<sup>7</sup> and Whitby and Hynes<sup>8</sup> improved upon his technic Maragliano<sup>9</sup> (1885 et seq) studied the resistance of erythrocytes to the action of heat, compression, exsiccation and human serum

Hamburger,<sup>10 11</sup> (1887 et seq) originated the most widely used method, which was modified a number of times by himself and later by others He used test tubes containing solutions of sodium chloride which varied in strength from 0.30 to 0.60 per cent, adding defibrinated blood which was measured by drops The interpretation of the test was based on the discoloration of the supernatant fluid by free hemoglobin Ribierre<sup>12</sup> wrote a comprehensive critical review in 1903 summing up the progress made in the preceding thirty years He modified Hamburger's technic by standardizing many details

In the ensuing years, many more modifications of Hamburger's methods were introduced (Mosso,<sup>13</sup> Viola,<sup>14</sup> Widal, Abramí and Brulé,<sup>15 16</sup> Ponder,<sup>17 18</sup> Wiseman and Bierbaum,<sup>19</sup> Lepeschkin,<sup>20</sup> Daland and Worthley,<sup>21</sup> Beebe and Hanley,<sup>22</sup> Creed,<sup>23</sup> Waugh and Asherman,<sup>24</sup> Dacie and Vaughan,<sup>25</sup> Hunter,<sup>26</sup> Berk,<sup>27</sup> Parpart<sup>2</sup>) Waugh and Chase<sup>29</sup> in 1928 described a method which combined the principle of counting unhemolyzed red cells in the hypotonic solutions-red cell mixture with the principle of measuring the amount of hemoglobin in the supernatant solution

The following are the most important features which have been modified by different authors

- 1 The type of anticoagulant used at first, defibrinated blood or fresh blood without an anticoagulant was used, later, oxalated, and still later, heparinized blood came to be preferred

- 2 The use of whole blood or washed red blood cells

- 3 The amount of blood and hypotonic solutions employed

- 4 The chemical composition, the number and strengths of the hypotonic solutions chosen Although most workers used solutions of sodium chloride, others selected solutions of other salts, such as sodium sulfate Simmel<sup>7</sup> used six different salts, attempting to create a mixture similar to that existing in the blood Other investigators used serum from the same patient diluted in different proportions with distilled water<sup>19</sup>

- 5 The method by which the hypotonic solutions and blood were measured, many authors still using the drop as the unit for either or both solutions and blood, others using carefully standardized pipets

- 6 The method for determining the beginning and the end of hemolysis and the percentage of hemolyzed red blood cells in various dilutions of hypotonic solutions Gallerani<sup>30</sup> used the Fleischl hemometer to determine the percentage of hemolyzed erythrocytes In an attempt to obtain more accurate results, Ponder devised differ-

ent methods to determine the degree of hemolysis he first used a radiometer,<sup>17</sup> later selenium<sup>18</sup> and potassium photoelectric cells, and still later a Stufenphotometer.<sup>31</sup> Waugh and Asherman<sup>24</sup> were apparently the first to use a photoelectric colorimeter for the measurement of the amount of hemoglobin present in the supernatant fluid in each tube.

7 The method of reporting results and, by some authors, of plotting them in graphic form. Many of the methods require the simultaneous performance of a control test from a known normal subject.

With various changes in method, many different results have been obtained so that the data of any one author for normal and pathologic conditions are seldom comparable to those of another. In most instances, all one can say is that the hypotonic resistance of the red cells is normal, increased or decreased.

## II. METHOD

### A. Photoelectric determination of the degree of hemolysis

Our method is a modification of the photoelectric determination of erythrocyte fragility, as first described by Waugh and Asherman<sup>24</sup> in 1938, and later modified by Hunter<sup>26</sup> in 1940. The Evelyn technic for the determination of oxyhemoglobin is used. Since it is known that hemolytic systems are affected by small changes in the pH, temperature, oxygen and carbon dioxide contents, and by the age and the concentration of the red cells, we have attempted some standardization of these variants.

The stock solutions are prepared from freshly distilled water and chemically pure sodium chloride, using the analytic balance. Buffer salts are purposely omitted because the sodium or potassium ions present may affect the tendency of the erythrocytes to hemolyze. Solutions are prepared at percentage intervals of 0.04 between the concentration of 0.04 per cent and 0.20 per cent and between those of 0.52 per cent and 0.80 per cent sodium chloride. Between the concentrations of 0.20 per cent and 0.52 per cent the percentage interval between solutions is 0.02 per cent. The solutions are kept in glass stoppered bottles and the pH of sample solutions checked at two week intervals by the Beckman pH meter. The pH of the distilled water and the solutions was found to range between the two extremes of 5.5 and 7.0. When a variation from these two extremes occurred new solutions were prepared. The saline concentration of the solutions is checked by the silver nitrate method to insure its correctness. Solutions are made up in 500 cc. volume every four to six weeks, more frequently in the summer than in the winter, to compensate for greater evaporation, and are kept at room temperature. A series of 29 conical centrifuge tubes is set up in consecutive order, from 0 (i.e., distilled water) to 0.80 per cent NaCl. Using the same pipet, 10 cc. of each solution is transferred into the appropriately labeled centrifuge tubes. Errors resulting from dilution or concentration of solutions are minimal since these are pipetted always in the same order (see below) and a difference from one solution to the next is too small to result in a significant error.

From 2.5 to 5 cc. of blood are collected without stasis from a suitable vein, and

sufficient heparin is used to prevent coagulation for 4 hours \* The vial of blood is gently rotated to insure complete oxygenation Exactly 0.02 cc of whole unwashed blood is delivered into each solution of NaCl by means of a standardized and calibrated Sahli 20 cu mm hemoglobin pipet After the blood has been pipetted into the salt solution, the pipet is filled once again with blood, which is discarded, and then again filled with blood for the next tube of salt solution

This is done in order to avoid hemolysis or dilution of the blood sample which might result from the fact that the pipet had become 'contaminated' by the salt solution It is advisable, when pipetting both the solution and the blood, to do so always *in the same order* so that whatever error results from lowering or raising the saline concentration will always be in the same direction in all tests Accordingly, we have pipetted the salt solutions by proceeding from the lower to the higher concentrations to avoid raising the saline concentrations, and we have pipetted blood by proceeding from the tubes in which no (or lesser) hemolysis is expected to occur to tubes in which greater hemolysis probably will take place

The tubes are inverted several times, left for one hour at room temperature, and then centrifuged at 1500 r p m for ten minutes The supernatant fluid from each test tube is carefully decanted and read in the Evelyn colorimeter for hemoglobin content, using filter No 540 Two perfectly matched absorption cells are used for all the readings The first cell contains the supernatant fluid from the 0.80 per cent tube, which is used as the blank, since no hemolysis usually occurs in this dilution, and the second cell is used to match the consecutive dilutions against the blank If there is any reason to suspect that hemolysis will take place, or has taken place at 0.80 per cent NaCl solution, a higher concentration of salt solution, 1 c, 0.85 per cent NaCl solution or 0.90 per cent, may be used for the blank

Bile pigments, or any other plasma constituents which might alter the light absorption, will not interfere with the reading since they are present in the blank in the same amount as in all the other tubes In any event, the use of the appropriate filter automatically eliminates the possible error deriving from the presence of chromogens other than hemoglobin

Several of the technics thus far devised include a 'correction' for anemia, which involves the use of a larger amount of blood or removal of a certain amount of plasma whenever anemia is present in order to include the same volume of red cells In our experience, fragility curves in both normal and abnormal subjects, using different amounts of blood, yielded curves with irregular variations which were within the limit of experimental error Alterations of erythrocyte fragility were by no means proportional to the degree of severity of the anemia Bloods with the same degree of anemia from patients with different conditions showed distinctly different types of curves For these reasons, and since the results of our tests are reported in terms of percentage of the total amount of red blood cells hemolyzed, 'correction' for anemia did not appear necessary and was, therefore, not carried out

\* One drop of Roche Organon Heparin (Liquaemin) as delivered from a No 20 needle is used for each sample of blood

The use of washed red blood cells rather than of whole, fresh, unmodified blood has been a highly controversial issue since Widal, Abram<sup>1</sup> and Brulé<sup>16</sup> showed that increased hemolysis to hypotonic salt solutions could be demonstrated in some patients whose unwashed erythrocytes yielded a normal type of fragility. Several investigations later indicated that washing the cholesterol off the red blood cells surface, as with a solution of sucrose, would result in an increase of erythrocyte fragility, whereas the removal of lecithin with Brinkman's solution would result in an increased resistance. The cholesterol lecithin ratio was considered as very important in this regard.

The above results could not be duplicated by other workers, who were unable to find any alteration in red cell fragility by washing erythrocytes with physiologic solutions (Saslow<sup>32</sup>). Ponder and Saslow<sup>33</sup> established that washing rabbits' red cells with sodium chloride or glucose solutions isotonic with rabbits' plasma did not alter the volume of red blood cells. They stated that the concentration of such solutions should be of 1-12 per cent NaCl.

Ponder<sup>34</sup> studied the inhibitory effect of blood serum on hemolysis, but these investigations were performed on fragility to hemolytic substances like saponin, bile salts and sodium hydroxide, where an interaction between the hemolytic agent and various plasma components could be expected. These results therefore could not be applied to hypotonic salt fragility.

In our laboratory, parallel fragilities performed on whole blood and on washed red blood cells showed somewhat less resistance in the latter, perhaps because 0.85 per cent sodium chloride which was used for washing may be regarded as a slightly hypotonic solution. We finally concluded that the use of whole unmodified blood was preferable to that of "washed" blood since it eliminated one step of doubtful value and since "washing" might actually injure the red cells, thus making them hypotonically fragile. In certain cases of hemolytic anemia, greater erythrocyte fragility might possibly be demonstrated with washed than with unwashed red cells. This fact was first stressed by Widal, Abram<sup>1</sup> and Brulé. As yet we have been unable to confirm their results, although further studies on this point are in progress.

Variation in the fragility of the same subject from day to day was found, but this was never of sufficient magnitude to give a significant difference in the interpretation of the results. This confirms the findings of Whitby and Hynes<sup>8</sup> in 1935.

#### *B Method for plotting the curve of hemolysis*

By plotting the per cent of hemolysis along the ordinate, against the decreasing strengths of saline along the abscissa, a sigmoid curve results. From inspection of the fragility curves as obtained by our method and of those of other investigators, it was noted that definite irregularities occurred in the rate of progression of hemolysis from tube to tube no matter how carefully the tests were performed. It was believed that in certain pathologic conditions these irregularities might represent changes in the "thickness population" of the blood, not well demonstrated by the ordinary methods in use for graphic charting.

A method was therefore devised in which the "hemolytic increments," were calculated and charted. This was done by determination of the additional amount of hemolysis occurring in the successive tubes of hypotonic salt solution which

contained progressively diminishing saline concentrations. For example, if no hemolysis occurs at 0.56 per cent sodium chloride, and 10 per cent of the red blood cells hemolyze at 0.52 per cent, and 50 per cent of the red cells hemolyze at 0.50 per cent, the hemolytic increment (i.e., the increase in degree of hemolysis) is 10 in the 0.52 per cent solution and 40 in the 0.50 per cent solution. The hemolytic increments, when plotted, yield curves that are somewhat similar in configuration to those obtained with Price-Jones curves of red cell diameters. They are considerably more graphic and informative than those obtained with the ordinary methods for plotting photoelectric fragility.

The actual figures for drawing the new type of curve are obtained in the following manner:

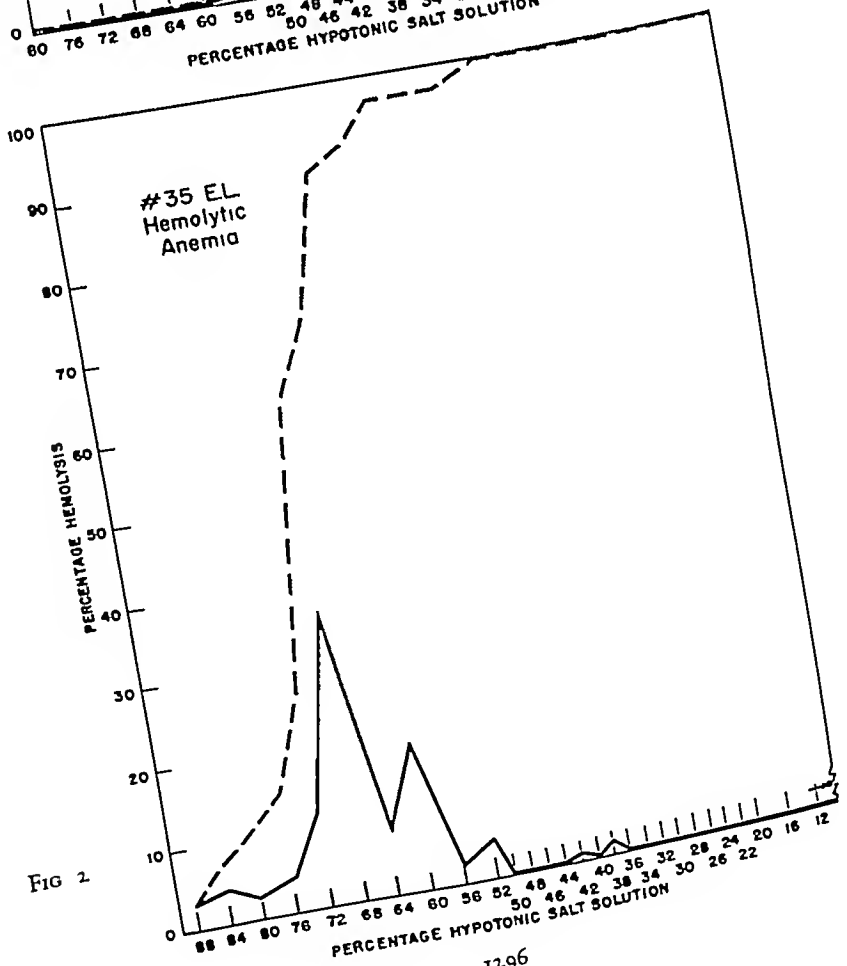
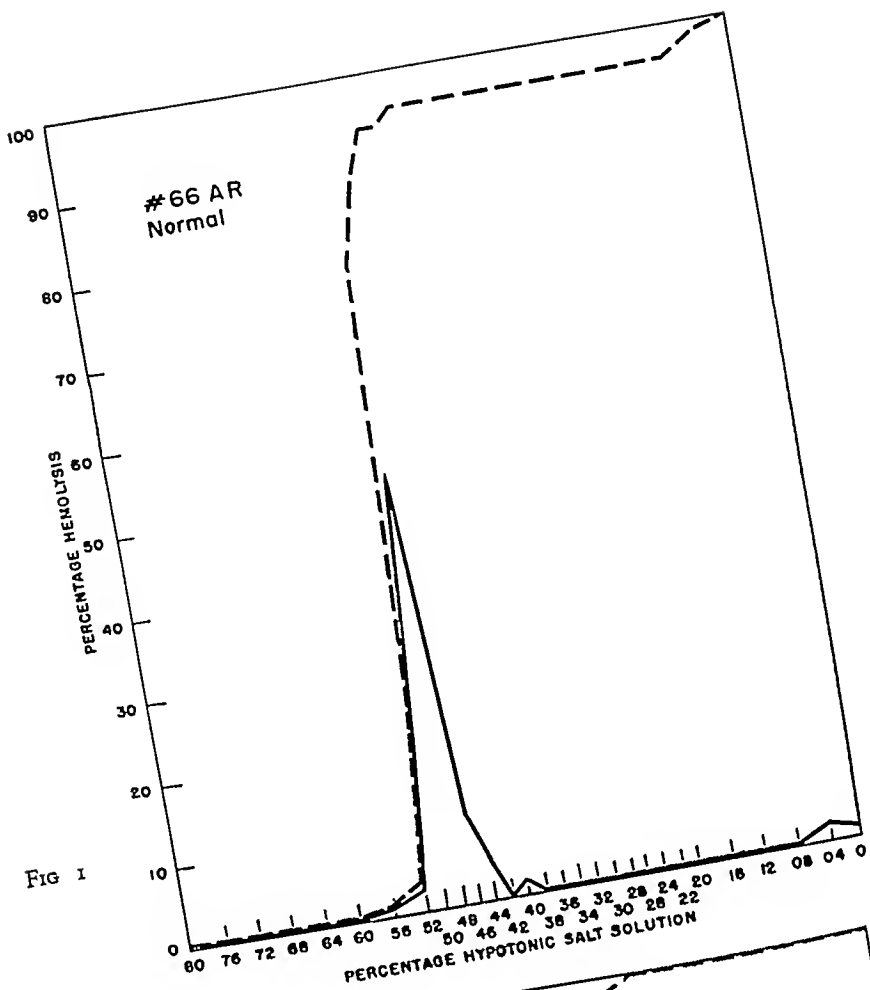
1. By using conversion tables, change the photometer readings into grams of oxyhemoglobin liberated.
2. Calculate the per cent of blood hemolyzed in each tube by dividing the hemoglobin reading in grams in the 0 tube (i.e., the total amount of hemoglobin) by the hemoglobin reading in grams in each tube.
3. Find the hemolytic increment by subtracting the percentage of blood hemolyzed in the first tube from that of the second, the second from the third, etc.
4. Draw a curve, plotting the percentage saline solutions on the abscissa and the hemolytic increments on the ordinate.

By proceeding from the observer's left to his right along the curve, it is seen that by adding the value at any one point to the values of all previous points (left to right) we obtain the total amount of red cells which have been hemolyzed at that concentration of sodium chloride. In other words, the value of the percentage of red blood cells hemolyzed, appearing in the curve at *each* concentration of sodium chloride expresses only the percentage of red blood cells which was hemolyzed at that particular concentration of sodium chloride and which had not been hemolyzed in the previous solutions.

If practically all cells tend to hemolyze at one point (isohemolysis) a sharply peaked monophasic curve results but if, on the other hand, the cells are hemolyzed over a wide range of hypotonicity (anisohemolysis) a biphasic or multiphasic curve results. The data obtained from determinations in both normal subjects and in those with various types of blood dyscrasias, revealed that certain diseases followed a more or less definite pattern with respect to the shape of the plotted curve of hemolytic increments. It appeared probable that the curves gave a graphic picture of the red blood cell thickness population, at least with the thickness diameter ratio as a principal factor.

In addition to the graphic representation of the hemolytic increments the following features have appeared to us to be of significance: (1) the concentration of the solution which shows beginning hemolysis, (2) the solution in which hemolysis is complete (since in a number of cases, both normal and pathologic, 100 per cent of the red blood cells hemolyze only in distilled water, we have concluded that the solution in which 90 per cent of the red blood cells hemolyze is a more significant index of total hemolysis), (3) the solution in which there is 50 per cent hemolysis, this solution being defined as indicating the mean corpuscular fragility, (4) the breadth of the curve, (5) the height of the highest hemolytic increment.





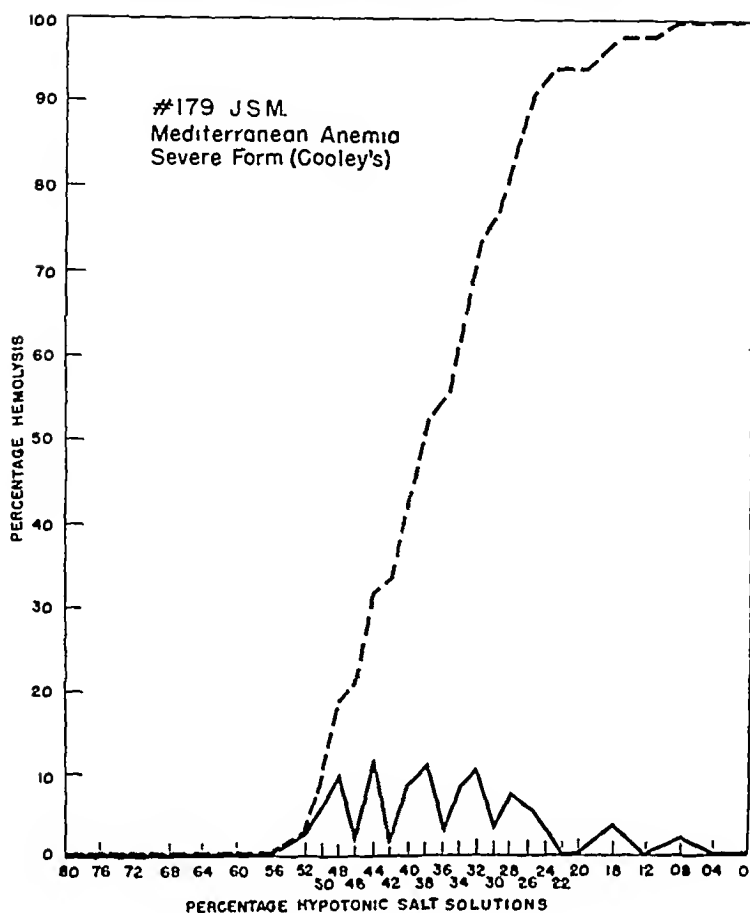


Fig 3

FIGS 1, 2, 3 PHOTOELECTRIC HYPOTONIC FRAGILITY CURVES IN A NORMAL SUBJECT, A SUBJECT WITH HEMOLYTIC ANEMIA, AND A SUBJECT WITH SEVERE MEDITERRANEAN ANEMIA

The curves are charted both by the conventional method and by that using hemolytic increments. The *first* curve (normal) is tall, monophasic and narrow based with most hemolysis occurring between concentrations of 0.52 and 0.44 per cent NaCl. The *second* curve, (hemolytic anemia) is broader and with more than one peak. Hemolysis begins in physiologic salt solution and is practically complete at 0.52 per cent NaCl. The *third* curve (Mediterranean anemia) is low, broad and multiphasic with hemolysis beginning at 52 per cent NaCl, complete very close to distilled water.

Normal individuals almost invariably yielded monophasic curves. The breadth of the curve was less than 20 per cent, that is, most of the red blood cells (90-95 per cent) hemolyzed within solutions differing by only 20 per cent sodium chloride. None or very little hemolysis occurred in the 0.60 per cent solution. Less than 5 per cent hemolysis occurred in the 0.56 per cent solution.

The highest hemolytic increment occurred normally in one of the salt solutions between 0.52 and 0.44 per cent and represented 40 to 50 per cent of the red blood cells. The mean corpuscular fragility (the solution in which 50 per cent RBC hemolyzed) was normally at 0.50 or 0.48 per cent. Ninety per cent of the red blood cells hemolyzed above the 0.42-0.40 per cent solutions. As already stated in many subjects, both normal and pathologic, a further increase in hemolysis occurred at or close to the distilled water level. While no explanation for this can be offered, one can speculate that this is due to the presence of a certain number of "new" red cells, i.e., reticulocytes, which because of their unusual thinness are unusually resistant to hypotonic solutions.

Figures 1, 2 and 3 are examples respectively of a normal curve, a curve

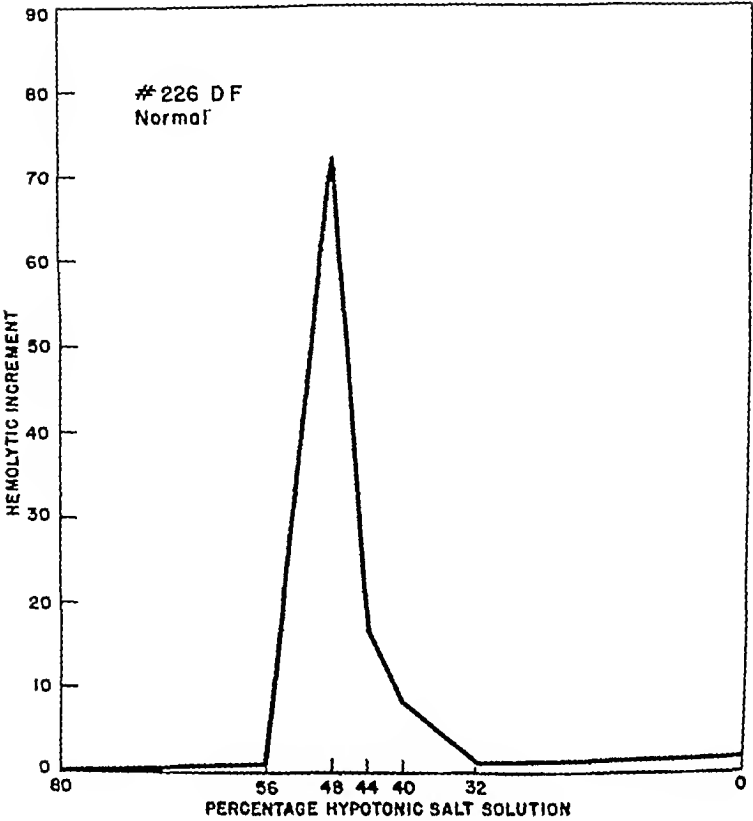


FIG 4

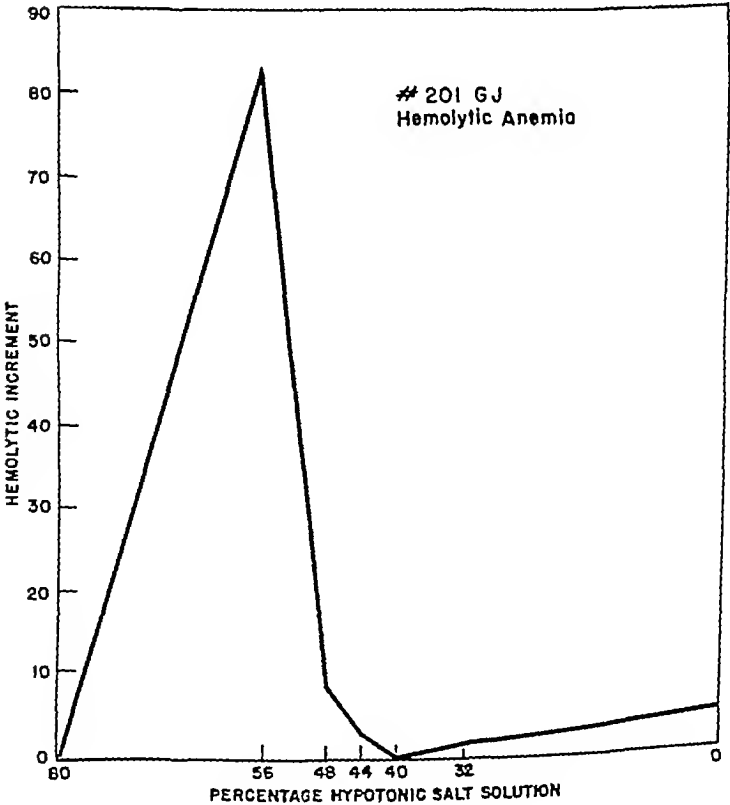


FIG 5

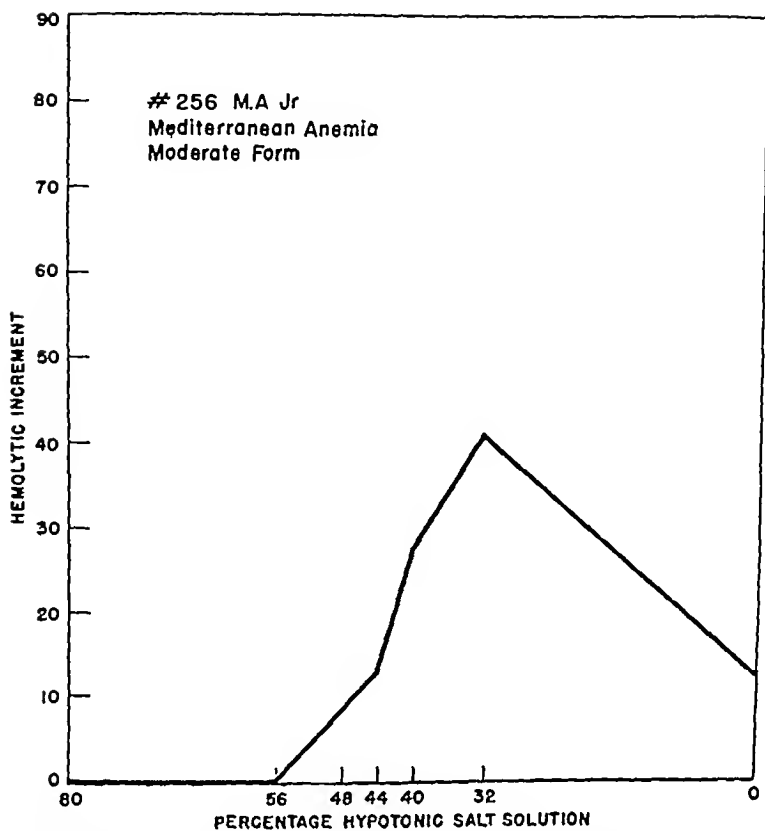


FIG 6

FIGS 4, 5, 6 PHOTOELECTRIC HYPOTONIC FRAGILITY CURVES WITH THE SIMPLIFIED (7 TUBE) METHOD IN A NORMAL SUBJECT, A SUBJECT WITH HEMOLYTIC ANEMIA AND A SUBJECT WITH MEDITERRANEAN ANEMIA

The curves show the range of hypotonic salt solutions in which hemolysis occurs with the typical shift to the right (leptocytosis) and to the left (spherocytosis) in the abnormal cases. These curves, although sufficient for most clinical diagnostic work, fail to give a detailed picture of the red cell population.

case of acquired hemolytic anemia, and a curve from a case of severe Mediterranean (Cooley's) anemia. The normal curve shows all of the above mentioned characteristics, i.e., the level at which hemolysis first takes place, the width of the base of the curve, and the height of the highest peak. The conventional type of hemolysis curve is also given for comparison. The curve from the case of hemolytic anemia shows a definite shift to the left (i.e., increased hemolysis in relatively concentrated solutions of NaCl) and a tendency to a very high peak as well as to a rather narrow base. The third curve, a severe case of Mediterranean anemia, shows a markedly broadened base, extending practically from the level at which hemolysis first begins in a normal subject, down to the distilled water level. The mean corpuscular fragility and the point at which 90 per cent of the red blood cells hemolyze shows a marked shift to the right. There are no high peaks but many small ones, indicating not only a great diversity in red cell thickness but indirectly a marked fault in the production of red blood cells of uniform degrees of thickness.

## III SIMPLIFIED METHOD

After eighty cases had been performed in both normal and abnormal subjects, a simplified test for clinical purposes was devised. In this test the following solutions were used 0.80, 0.56, 0.48, 0.44, 0.40, 0.32 per cent NaCl solution and distilled water. The 0.80 per cent solution was used as a blank since hemolysis of both normal and abnormal bloods did not usually occur in this solution. For the determination of total hemoglobin, distilled water was used. In the 0.56 per cent solution, normal subjects showed less than 5 per cent red blood cell hemolysis, a much higher percentage of hemolysis in this tube occurred in the various types of hemolytic anemia. The 0.48, 0.44, and 0.40 per cent solutions showed the greatest degree of hemolytic increment in normal subjects, 16, 45 to 75 per cent, 15 to 35 per cent, and 25 to 15 per cent respectively. The 0.32 per cent saline gave hemolysis of close to 100 per cent red blood cells in normal subjects, but of lesser degree in the abnormal states with increased hypotonic resistance.

The results of this simplified method were plotted in curves in the same manner as outlined above for the complete test. Figures 4, 5 and 6 are examples of 3 curves obtained with the simplified "seven tube" method in a normal subject, a case of hemolytic anemia and a case of Mediterranean anemia, respectively. The different characteristics of the curves are obvious at a glance, with the shift to the "left" in the case of hemolytic anemia, and the shift to the "right" in the case of Mediterranean anemia.

Several simplified methods, utilizing only two to four tubes, have been devised in the past for the purpose of obtaining prompt information as to the fragility of the red blood cells (Lepeschkin,<sup>20</sup> Berk,<sup>27</sup> and Smith<sup>36</sup>). The use of seven tubes appears to be simple enough to allow for relatively quick performance. It is obvious, however, that it does not give the regular types of curves seen with the complete test, nor details as to the variations in red cell population. It thus appears to be unsatisfactory for investigative purposes, although as a clinical test it appears to have definite merit.

## IV DISCUSSION

Ponder<sup>31</sup> made use of two types of curves in his extensive studies. He used the term "time dilution" curve to designate the curve obtained by plotting the time required for *complete* hemolysis against each dilution of the lysis used. The percentage of red cells hemolyzed was plotted against time in the second type of curve which he called "percentage hemolysis." He was more interested in hemolytic agents such as saponin, and sodium taurocholate, than in hypotonic salt solutions. Besides, he always introduced the element of time for the hemolytic reaction to occur, and his investigations were for the most part limited to normal erythrocytes. Unfortunately, his contributions have not as yet found wide application in the clinical field.

Various investigators have questioned the importance and value of the shape of the curve obtained when quantitative methods for determining hemolysis are used. Thus Vaughan<sup>37</sup> expressed the belief that the curve of hemolysis shown by plotting the concentration of saline against the degree of hemolysis, both being

expressed as a percentage, utilizes a method which is clearly unsatisfactory, since markedly different curves may lie in the same range." Creed<sup>23</sup> in 1938 wrote "it was hoped that the form of a fragility curve constructed from the data obtained might show distinctive differences in various blood diseases and possibly in other conditions. For the most part these hopes have not been realized." He also stated that there was a slight difference in the shape of the curve in pernicious anemia.

According to Dacie and Vaughan,<sup>26</sup> "the shape of the curve or span of resistance is without great practical significance," and "study of the shape of the curve in pathological bloods is not in practice very useful." In a later publication, however, Dacie<sup>25</sup> paid more attention to the shape of the curves, classifying them as 'tailed,' 'diagonal' and of 'normal type.' Berk<sup>27</sup> agreed with the above statements of Dacie and Vaughan.

Waugh and Asherman,<sup>24</sup> as well as Hunter,<sup>26</sup> plotted their results in curves. However, Waugh and Asherman did not attach much importance to them. Hunter wrote "If the technique has been carefully carried out the curve will ordinarily be sigmoid in character. In some instances, particularly in the case of blood showing increased fragility, the curve may show more than one maximal slope. The significance of this occasional variation from the usual curve, however, is by no means clear, and requires further investigation."

Our results indicate that the shape of the curve obtained, particularly as modified by the use of the hemolytic increment method, is of great importance. With our method of plotting, one can readily distinguish between bloods showing spherocytosis and bloods containing target (1 e, thin) red cells. What is perhaps more important is recognition of the concept that the changes in hemolytic increment from tube to tube indicate the hemolysis of different groups of red cells, i.e., differences in red cell population thickness. This in turn might indicate either different types of red cells from the standpoint of their production by the bone marrow or differences in the ages of groups of red cells, since it is our belief that the cells just delivered from the bone marrow, i.e., the reticulocytes, are thinnest, and the oldest red cells the most spherocytic. The reason for the fragility "range" even in normal subjects is in all probability due to this age difference in red cells, the oldest ones hemolyzing at 0.56 per cent or thereabouts, the youngest at 0.40 or 0.42 per cent.

In normal subjects, a very sharp and regular type of curve is present indicating a relatively uniform type of red cell and of age thickness population. 'Little bumps' in the curve, particularly in the more hypotonic section of the curve, may be due to the presence of a certain number of relatively thin reticulocytes.

In subjects with Mediterranean anemia, the marked irregularity in the curve of hemolytic increments from tube to tube almost certainly indicates a marked variation in the type of red cell produced by the marrow. This brings graphically to light the fault in the red cell (and hemoglobin) production which appears to be at the basis of the disease. Thus, a careful reading of the hemolytic increment curves permits some insight not only as regards the variations in red cell population in given cases but also into the pathologic physiology of red cell formation.

itself. The use of the terms 'isohemolysis, anisohemolysis, shift to the left, shift to the right, broad base, etc.' appear to be of some value in describing the type of hemolytic curves obtained by this method, and thus in developing further insight into the physiopathologic mechanisms of blood destruction.

## V. SUMMARY

A method is described for the determination and charting of the fragility of the red blood cells to hypotonic sodium chloride solution. For plotting the results obtained, a new method was used, employing the principle of "hemolytic increments," i.e., plotting the additional amount of hemolysis occurring in each successive tube of solutions of decreasing saline concentration.

By this method, curves are obtained somewhat similar to those of Price-Jones curves of red cell diameters. Such curves, when properly analyzed, afford an interpretation of the varying diameter thickness range of the red cell population and indirectly an insight into the types of red cells present, and of the possible hemolytic mechanisms present. Preliminary investigations indicate that they are particularly instructive in the hemolytic anemias and especially in the Mediterranean anemias.

A simplified shorter method was also devised for routine clinical use. This method, although not as useful for research purposes, has proved to be more accurate and yet less time consuming than most methods currently used.

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## EDITORIAL

### HEPARINEMIA

EARLY workers on blood coagulation often used the terms anticoagulant and antithrombin interchangeably. Even today this tendency is still evident, especially in the writings of those who are more familiar with the chemical than the physiologic aspects of blood coagulation. Antithrombic substances are, of course, anticoagulants, but there are several anticoagulants which have no antithrombin activity. The first efforts designed to demonstrate the presence of heparin in the blood depended on the effect of addition of extracts of blood on the rate of coagulation and antithrombin activity of normal blood. The results were not striking.<sup>1</sup> When Chargaff<sup>2</sup> showed that protamine combined with heparin quantitatively, the way was open for an accurate method for the titration of heparin in the blood. However, the smallest amounts of protamine that can be added to normal blood and still produce an effect usually delay coagulation rather than accelerate it. The same may be said for toluidin blue and similar heparin-binding dyes. Jaques and Waters<sup>3</sup> were unable to recover any heparin from as much as 4 liters of blood of normal dogs.

Moreover, heparin in low concentrations actually accelerates the coagulation of blood. This effect, first brought out by Fischer,<sup>4</sup> has been confirmed both with natural heparin<sup>5</sup> and a synthetic substitute.<sup>6</sup> The amounts of natural heparin necessary are of the order of 0.001 to 0.01 mg per 100 cc of blood, well beyond the range of detection by protamine titration methods. There are no figures available regarding the concentration of heparin in the blood of man or intact experimental animals.<sup>7</sup> Likewise, no heparin (or any other anticoagulant) has to date<sup>8</sup> been found among the fractions separated from human plasma by the Cohn method.

It seems that, normally, heparin is contained within the mast cells of the tissues.<sup>7</sup> Drastic cytolytic agents, such as are employed in its preparation, are necessary to release it from the cells.<sup>9</sup> This probably explains why the blood of animals with neoplasms containing large aggregations of mast cells (mast cell tumors of dogs)<sup>10</sup> displays no altered coagulation and contains no heparin, despite the fact that the yield of heparin from extracts of such tumors in the dog is fifty times that of extracts of the liver.<sup>10</sup>

The foregoing throws some doubt on the existence of heparin as a component of normal blood, or as its physiologic anticoagulant, but provides a basis for judging the significance of recent reports of the presence of heparin in the blood of man and experimental animals. *Heparinemia* would seem to be the proper designation for such a phenomenon.

In animals, heparinemia has been observed after intravenous injections of peptone<sup>9</sup> ascaris extracts and trypsin<sup>11</sup> in anaphylactic shock<sup>12-13</sup> and after exposure of the body to massive ionizing radiation.<sup>13-15</sup> These experimental findings made it seem highly probable that heparinemia might occur in man. A recent report of the presence of a heparin-like anticoagulant in the blood of patients treated with one

of the nitrogen mustards seems to be the first *convincing* demonstration of such an occurrence in man <sup>14</sup> No doubt, a greater awareness of such a possibility, and familiarity with the special technics necessary for the demonstration of heparin will lead to its being found associated with other clinical states. Heparin may conceivably be liberated during the explosive phase of certain severe systemic reactions, especially those involving widespread cytolysis. The unexpected, urgent, transient and fatal character of these reactions in man has made them, in the past, unsuitable for careful study.

Until lately, <sup>14</sup> spontaneously developed heparinemia did not constitute a clinical challenge. In patients receiving heparin because of thromboembolic disease, excessive bleeding occasionally has made it desirable to restore the coagulation of the blood to normal. Protamine injected intravenously (1 - 2 mg /Kg body weight) does this effectively, since this basic protein binds the acidic heparin quantitatively in a proportion of 2 to 1.

It is possible that spontaneously developed heparinemia may sometime become an important clinical entity and demand similar management. In the hemorrhagic phases accompanying acute leukemias, thrombopenias and aplastic anemias, some not altogether conclusive evidence has been offered that heparinemia exists <sup>15</sup> Therapeutic trials with protamine and toluidin blue (an antiheparin dye) have been made. In contrast with the experimental heparinemias following radiation, no convincing *direct* evidence has been brought out of the presence of heparin in the blood of patients with leukemia, thrombocytopenia and aplastic anemia. In our hands, protamine therapy has not altered significantly the hemorrhagic manifestations in these states.

Heparinemia should be looked for in clinical states accompanied by chronic widespread tissue necrosis. Conditions resembling those brought about by ionizing radiations may conceivably be produced by other pathogenic agents. However, anticoagulants other than heparin may be found in such states. Evidence has recently been advanced that entrance of anticoagulant substances into the blood or an increase in those already present therein <sup>16 17</sup> will alter the finely adjusted balance between anticoagulant and coagulant factors, to which the blood owes its fluidity and coagulability. Up to relatively recent times, absolute deficiencies in the coagulant factors (platelets, prothrombin, fibrinogen) were almost exclusively held responsible for diminutions in the coagulability of blood. The recently accumulated facts have served to modify this stand and to support what seems to be a more rational, though not as simple, explanation of the mechanisms underlying the maintenance of the fluidity and coagulability of circulating blood.

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# ABSTRACTS

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ABSTRACTERS

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## ANEMIA

EXPERIMENTAL FINDINGS IN LATENT SIDEROPENIC ANEMIA *E. Rothblum and E. Undritz* From the laboratory for experimental pharmacology of Sandoz S. A., Basle, Switzerland *Schweiz med Wschr* 1947 58-60

Sideropenic anemia is known as a definite disease. Experimentally the syndrome has been produced in the white laboratory rat and, as in human patients, was shown to respond to iron therapy. The authors found that normal hemoglobin findings do not always correspond to normal iron depot.

C. M.

DEVELOPMENT OF PERNICIOUS ANEMIA IN A CASE OF MICROCYTIC HYPOCHROMIC ANEMIA *F. Fierz* From the Medical Section of the Cantonal Hospital, Aarau, Switzerland *Schweiz med Wschr* 1947 1198-1199

A female patient with nanism of pituitary origin was under observation for hypochromic microcytic anemia. She developed during pregnancy by exhaustion of the intrinsic factor the hematologic picture of pernicious anemia.

C. M.

FOLIC ACID THERAPY: RESULTS OF A CLINICAL STUDY *W. S. Adams and J. S. Lawrence* From the Department of Medicine, University of Rochester School of Medicine and Dentistry and Medical Clinic of the Strong Memorial and Rochester Municipal Hospitals, Rochester, New York *Am J M Sc* 215 487-497, 1948

Thirty-four miscellaneous cases treated with folic acid are reported and correlated with other reports in the literature. The authors conclude that folic acid is effective in macrocytic anemia with a megaloblastic marrow, and in sprue. No effect was obtained in the anemia of chronic nephritis, leukopenic of Addison's disease or Felty's syndrome, idiopathic leukopenia, leukemia or leukopenia following irradiation. Again the statement is made that folic acid does not prevent the progression of the neurologic manifestations of pernicious anemia.

C. A. F.

## LEUKOCYTES AND LEUKOCYTIC DISEASE

THE LEUCOCYTOSIS OF DIABETIC ACIDOSIS *J. L. Tullis* From the Department of Biological Chemistry, Harvard Medical School, and the Laboratories of Pathology, New England Deaconess Hospital, Boston, Mass. *Am J M Sc* 215 424-426, 1948

The author has extended his interesting studies on the effect of variation in the tonicity of blood on the circulating leukocytes. In seven patients in diabetic coma, levels of hypertonicity were determined by freezing point depression measurements on defibrinated blood and compared with changes in circulating leukocytes. These patients showed a variation in molarity of their blood from 344 to 349 millimoles per liter on admission and after recovery a variation from 301 to 349 millimoles. White counts varied from 18,700 to 26,760 per cubic millimeter. There was a linear relationship between molarity and absolute numbers of circulating neutrophils. It is of interest that there were no eosinophils seen in the

smears of these patients when in coma, and that the same alteration in leukocyte pattern is seen in conditions of stress and after adrenal cortical stimulation. The author, however, has previously shown that these changes occur in circumstances where the adrenal cortex mechanism is not involved

C A F

REMARQUABLES RESULTATS DU TRAITEMENT PAR L'EXSANGUINO-TRANSFUSION D'UN CAS DE LEUCEMIE AIGUE (Remarkable Results of the Treatment of a Case of Acute Leukemia by Exsanguino-transfusion) *M. Bessis and J. Bernard*, Bull et mem Soc Méd d hôp de Paris 28, 29, p 871-877, 1947

The authors describe the case of a child, 6, with an acute leukemia with pyrexia, pallor, gum lesions, hemorrhages, hepatomegaly, splenomegaly, enlarged lymph glands, and with a typical blood picture 1,370,000 red cells, 86,000 white cells of which 98 per cent were lymphoblastic, and an abnormal bone marrow (99 per cent lymphoblasts). Transfusions were undertaken when the outlook was extremely grave and death appeared imminent. They were immediately followed by a marked and progressive improvement. In a few days the child's state was transformed, the physical signs disappeared and the blood and marrow returned to normal. The authors stress the following points: the diagnosis cannot be questioned. It was an absolutely typical acute leukemia, conforming to the classical descriptions. The rapidity and scale of the improvement are very remarkable. This child was moribund on admission to hospital. His state was so grave that the exsanguino-transfusion decided for the next day, was done during the night. From the first exsanguino-transfusion (only partial), the improvement was profound. After the second the child was transformed and, within a few days, apparently in excellent health. The progression of the improvement should also be noted. Not only was there a very marked improvement after each exsanguino-transfusion, but the maximum improvement was not reached at once. It continued during the days following the transfusion. This fact seems to counteract the idea that the treatment has only a palliative action. The final condition of the patient is worthy of attention: not only had the pallor, fever and hemorrhages disappeared, but also the liver, spleen and lymph glands diminished in size and after a few days became completely normal. There was also a progressive improvement of the blood and marrow. This favorable course was provoked by each exsanguino-transfusion. The first one allowed the patient to survive. The second caused a considerable regression of physical signs and a partial improvement in the condition of the blood and marrow. The third was followed by the return of the blood and marrow to normal. The authors do not believe that they have cured the child, but think that these early results allow hope that more will soon be known about the mechanism of the leukemia. The studies that they have undertaken may perhaps cause them to improve the method, possibly by the choice of special donors whose blood will contain the maximum quantity of the hypothetical antileukemic substance. It is possible too that the method will be applicable to other sarcomatoses, apart from the leukoses

J P S

THE SKELETAL LESIONS IN LEUKEMIA. CLINICAL AND ROENTGENOGRAPHIC OBSERVATIONS IN 103 INFANTS AND CHILDREN, WITH A REVIEW OF THE LITERATURE. *F. N. Silverman*. From the Department of Pediatrics, College of Physicians and Surgeons, Columbia University, and the Babies Hospital, New York City. *Am J of Roentgenol* 59 819-844, 1948

The authors have reviewed the reports in the literature of skeletal changes associated with leukemia. They selected 131 well documented cases of leukemia of which 103 had been adequately studied roentgenologically. Data as to signs and symptoms, laboratory findings, and clinical course are included. Fifty-two of the 103 patients showed bone lesions. These were of four types: (1) transverse bands of diminished density (14 patients), (2) osteolysis (39 patients), (3) osteosclerosis (9 patients), and (4) subperiosteal new bone formation (17 patients). Approximately one half of these had bone pain.

The differential diagnosis presented by these patients is discussed. While some of the more general material in this article is of an arbitrary nature, the article provides a comprehensive discussion of the value of the roentgenologic examination in childhood leukemia.

C A F

MECHANISM OF URETHANE IN LEUCAEMIA. *S. Moeschlin*. University Clinic of Internal Medicine, Zurich, Switzerland. *Helvet med Acta* 14 279-294, 1947

Observations in healthy subjects under therapeutic doses of urethane during 14-32 days did not show

any notable changes in bone marrow. In chronic myeloid leukemia no evident changes in cell composition were found. Calculation of the mitotic index in leukemia cells showed a decrease of mitotic activity. The favorable action of urethane in leukemia is based upon a selective suppression of mitosis in the pathologic myeloblastic cells.

C M

**A CASE OF FELTY SYNDROME WITH CYCLIC AGRANULOCYTOSIS** *W. Löffler and C. Matter* University Clinic of Internal Medicine, Zurich, Switzerland. *Cardiologia (Basle)* XII 195-210, 1947.

A case of Felty syndrome is described characterized by a peculiar agranulocytic bone marrow reaction. During clinical observation of three years the patient showed recurrent agranulocytosis in a three weeks cycle. Splenectomy had no effect.

C M

**TWO CASES OF TULARAEMIA TULARAEMIA AND INFECTIOUS MONONUCLEOSIS** *V. de Lavergne, G. Favre, L. Blanc and P. Boissel* Bull. et Mem. Soc. Med. d. hop. de Paris 189-193, 1948.

Three days after they had skinned a hare which they found dead, two subjects presented simultaneously the following symptoms: fever, headache, asthenia, anorexia, numerous enlarged, tender lymph-glands, especially in the neck and the trapezoid regions. The spleen was palpable in one patient and enlarged to percussion in the other. One of the patients developed conjunctivitis, the other enlarged tonsils.

The diagnosis of a *Pasteurella tularensis* affection was confirmed in both cases by serodiagnostic test positive to 1/75 and reaching 1/2000 on the 45th day) and by guinea pig inoculation of a portion of a lymph gland and of a positive pharyngeal swab.

In both cases, treatment by streptomycin showed itself remarkably efficacious—apyrexia within 72 hours, disappearance of all symptoms within 8 days and rapid resolution of the conjunctivitis.

The diagnosis was facilitated by the occurrence of two simultaneous cases and by the contact with a suspect hare. Had the case been isolated, or had one been unaware of the contact with a hare, the clinical diagnosis would doubtless have been that of infectious mononucleosis. In fact, the number of leukocytes in each case was 8,000, with only 43 per cent of polymorphs in the first case and 37 per cent of polymorphs in the second. In both cases 6-7 per cent of atypical lymphocytes were found among the mononuclears.

The really interesting point was the occurrence in both cases of a Paul and Bunnell reaction positive to 1/112 in one and 1/896 in the other.

The two observations are however still troubling. Although the authors have studied the agglutination of *P. tularensis* in six cases of infectious mononucleosis and have found it constantly negative, when one is faced by the clinical picture of infectious mononucleosis, the possibility of tularemia should be kept in mind and enquiries made as to possible sources of contagion (particularly game).

J P S

**MALIGNANT BONE AND BONE MARROW NEOPLASTIC TUMORS** *K. Rohr* University Clinic of Internal Medicine, Zurich, Switzerland. *Schweiz. med. Wschr.* 1947 207-215.

According to the authors' conception, leukemias are neoplastic manifestations of the hematopoietic organs. In the clinical course of these neoplasias he distinguishes a first phase of primary tumor, a second phase of hematogenic and lymphogenic generalization and, in leukemias, a third phase with propagation of cells in the blood stream. All bone and bone marrow tumors show early metastatic growth. In order to understand the biologic aspect of the different tumor types, it is necessary to consider the histogenesis. Besides the morphologic differentiation, certain physiologic aspects are of importance (increase of phosphatase in sarcomas, hyperproteinemia in multiple myelomas, increase of oxydase and porphyrins in leukemias). The author considers the reticulohistiocytic system as the genetic substrate for the myeloma cells and presumes a differentiation either towards plasma-cells or towards histiocytes. In leukemias he discriminates a myelocytic (chronic) variation, a promyelocytic (more subacute) and a myeloblastic (acute) variation based on the degree of morphologic differentiation of the cells. The myeloblastic leukemia more often has lymphogenic origin.

C M

PLASMOCYTOMA (MULTIPLE MYELOMA, KAHLE'S DISEASE) AND ITS ATTENDANT DISTURBANCES IN THE PROTEIN METABOLISM *W J Kolff and J Dhont* From the Municipal Hospital Engelenberg Foundation, Kampen, Holland *Am J M Sc* 215 405-410, 1948

The authors discuss a concept of plasmocytoma, based on recent articles in the Netherlands medical literature and on case studies. They have adapted Apitz's opinion that solitary plasmocytoma and diffuse plasmocytosis represent the same process. They question whether a local increase in plasma cells occurs and have always found a diffuse increase in plasma cells in cases of plasmocytoma. The importance of fresh preparations obtained either by sternal puncture or immediately after death in identification of plasma cells is stressed. They discuss the origin of Bence-Jones protein and feel that it is found specifically in this disease. Primary amyloidosis is felt to be a manifestation exclusively of plasmocytoma.

Some of these statements are open to question, in the opinion of the reviewer. A diffuse increase in plasma cells is found not infrequently in other diseases, and some caution must be exercised in making the diagnosis of plasmocytoma on such grounds. Likewise, one hesitates to accept the statement that all cases of primary amyloidosis are attributable to plasmocytoma.

C A F

## HEMOLYTIC ANEMIA

AN ANTI-RH ANTIGEN-ANTIBODY REACTION FACTOR (THE RH PROTECTIVE FACTOR) PRELIMINARY REPORT  
*A Bloxson and R Matthaei* *Bull Johns Hopkins Hosp* 82 1-9, 1948

The authors believe they have demonstrated the presence of a factor in sera from Rh negative individuals which partially inhibits agglutination of Rh positive cells by Rh agglutinating antibodies. It is suggested that this inhibiting or protective factor may be active in vivo when Rh negative blood is used in the treatment of hemolytic disease of the newborn, and may at times be present in the maternal circulation and exert an inhibiting effect on the disease in the fetus. The observations on which these important assumptions are made appear rather tenuous and need confirmation.

R S E

THE LYSOLECITHIN TEST IN HEMOLYTIC ANEMIAS *C Maier* University Polyclinic of Internal Medicine, Zurich, Switzerland *Helvet med Acta* 14 470-474, 1947

Following the technic of Singer, investigations in hemolytic anemia were conducted. In anemia with secondary hemolytic action the lysolecithin-resistance is normal. In a case of severe subacute acquired hemolytic anemia with reduced osmotic resistance, the lysolecithin-resistance was distinctly increased. In cases of constitutional hemolytic anemia the lysolecithin-resistance is reduced. The same findings have been obtained in a case of chronic thrombosis of the splenic vein.

C M

EXPERIMENTAL HEMOLYTIC ANEMIA *W Baumgartner* University Clinic of Internal Medicine, Berne, Switzerland *Helvet med Acta*, 14 502-507, 1947

According to the proceeding of Damashek and Schwartz the author produced hemolytic anemia of the immune body type in guinea-pigs. For the first time he found in the histologic examination of the spleen large macrophage cells storing erythrocytes. Antibodies are supposed to act not only as agglutinins and hemolysins but also as opsonins. He also found parenchymatic cells containing erythrocytes in the liver. Similar finding could not be obtained in cases of constitutional hemolytic anemia.

C M

THE CRYPTOGENIC ACQUIRED HEMOLYTIC ANEMIAS *J A Fisher* From the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford *The Quarterly Jr Med*, 16 245, 1947

Eighteen cases of hemolytic anemia are discussed under the three headings of miscellaneous, acquired hemolytic spherocytic jaundice, and hemolytic anemia with paroxysmal hemoglobinuria. The difference between the first two groups does not appear fundamental, since neither studies of longevity of transfused cells nor the Coombs test was used as a basis for separation. The Coombs test was found to be positive when done in one case. The report serves to stress further the infinite variety of patients with

hemolytic disease and the author makes 7 pertinent observation in the association of hemolytic anemia leukopenia, and thrombocytopenia, and states, It seems reasonable to postulate a similar rather than a dissimilar pathologic process to account for the deficiency in the other two components, the platelets and the neutrophils

R S E

A STATISTICAL STUDY OF THE HEMATOLOGIC VARIABLES IN SUBJECTS WITH THALASSEMIA MINOR *W N Valentine and J V Neel* From the Department of Medicine of the University of Rochester School of Medicine and Dentistry, Rochester, New York *Am J M Sc* 215 456-460, 1948

Differences in the peripheral blood picture between patients with *Thalassemia Minor* and control groups were studied. The difference was already apparent in childhood, and amounted to 2-3 grams hemoglobin per 100 cc blood. This difference was superimposed on the normal sex variation. The tendency to have an elevated erythrocyte count was also observed. While these data are not new, they serve to quantify the abnormalities in this condition.

C A F

THE SIGNIFICANCE OF THE PAUCITY OF SICKLE CELLS IN NEWBORN NEGRO INFANTS *J Watson, AW Stabman and F P Bilello* From the Department of Internal Medicine, Long Island College of Medicine Brooklyn, New York *Am J M Sc* 215 419-423, 1948

Sickle cell preparations were made on 226 consecutive Negro newborn infants and their mothers. There was an incidence of sickle cells of 8 per cent among the mothers and 8.4 per cent in the infants. A longer time was required to sickle the infants' erythrocytes, and infants' erythrocytes showed only 0.5 to 29.5 per cent sickling in contrast to 84-100 per cent in maternal blood. The authors suggest that this difference represents a chemical difference between fetal and adult hemoglobin. Sickling increased in one patient studied from 6-90 per cent over the first four and a half months, paralleling the expected disappearance of fetal hemoglobin. This focuses attention on the role of hemoglobin in the sickling process.

C A F

## BLOOD CLOTTING AND HEMORRHAGIC DISEASE

THE FIBRINOPENIC DIATHESIS. THE MAJOR PSEUDOHAEOPHILIC AFIBRINEMIA AND THE HYPOFIBRINAEMIAS. THE FIBRINOGEN LEVEL IN HAEMORRHAGIC STATES. *P Croizat, L Revol and J Favre-Gilly* Lyon, France *Sang* 19 26-35, 1948

The authors studied the fibrinogen levels in sixty cases with haemorrhagic syndromes. They distinguish three primary groups of hypofibrinemias.

(1) An idiopathic hypofibrinemic diathesis with a fibrinogen level between 1 and 2.5 gm (méthode pondézale) responsible for certain recurring hemorrhages, such as epistaxis, vitreous hemorrhages, metrorrhagia, etc.

(2) Hypofibrinemias of hepatic origin.

(3) Finally, the authors report three cases of pseudohemophilic afibrinemia with fibrinogen levels less than 0.5 gm. Clinically, this more or less total absence of fibrinogen may be suspected by the sign of the microclot.

This work has been developed in an important study by Favre-Gilly (Paris, Publ. Vigot Freres 1947) in which he deals with the whole problem of the fibrinopenias.

The only point which seems to us to need discussion, concerns the hypofibrinemias. A level of 2 gm of fibrinogen is often found in normal subjects. On the other hand, the fact that a fibrinogen level of 1.5-2 gm coincides with localized recurring hemorrhages does not seem to us to be proof that it is the cause of these phenomena.

J P S

STUDY OF THROMBOCYTES WITH THE ELECTRON MICROSCOPE. *M Bessis and M Burstein* Paris. *Rev d hemat* 3 48-68, 1948

The authors describe the technic of their examinations in order to observe the platelets in their cir-



culating form, as *in vivo*, they took the blood with a paraffined needle and put it directly into a citrate solution containing 1 per cent formol

In order to observe the transient resting forms, already described as seen under the ultra-microscope by Fonio and Schwedener, they put the blood into citrate or oxalate solution

In both cases the plasma is centrifuged for three minutes at a speed of 2000 r p m. One drop of the plasma is then spread on a slide and covered with collodion, before dissolving glass with hydrofluoric acid. The fine particles of collodion which are to be deposited on the objective of the electron microscope are then dispersed.

Under these conditions, the circulating forms appear perfectly round with a central, very dense, nucleus and occasionally several granules.

In the transitional or dendritic forms, one observes the dendrites which can be seen on many microphotographs (magnification  $\times 10,000$ ).

Finally, the resting forms appear as a layer of round cells with a very fine cytoplasm and a slightly condensed granular nucleus.

Goose thrombocytes were examined by the same technic.

The authors draw attention to the internal structure of the platelets which are formed of interlacing fibres around tiny spheres, and also to the little spherical bodies found in the plasma during the pre-coagulation period and of which the significance is still unknown.

J P S

# NEWS AND VIEWS

## INTERNATIONAL SOCIETY OF HEMATOLOGY

The International Society of Hematology in its first full dress Congress met at Buffalo, August 23-27. There were 680 registrants from 18 different countries, including large groups from the Latin American countries and several individuals each from Great Britain, France, Holland, Sweden, Denmark and Norway.

The meetings were fully attended and the numerous exhibits and papers proved to be of unusual interest. Those attending the meetings were highly enthusiastic not only because of the high calibre of the papers and the discussions but for the opportunity for face to face discussion with the various groups of workers in the field of hematology throughout the world.

The first day's program was devoted to hemorrhagic disease and included papers by Diamond (Boston) on Congenital Afibrinogenemia, Soulier (Paris), Prothrombin Consumption, Seegers (Detroit), Prothrombin Activation, Owren (Oslo) on Factor V, Lozner (Syracuse) and Pavlovsky (Buenos Aires) on Hemophilia, Macfarlane (Oxford) on Prothrombin, Tocantins (Philadelphia) on Antithromboplastin, Jacques (Saskatchewan) on Heparinemia, and Bernard (Paris) on Thrombocytic Dystrophy.

The second day's program dealt with erythropoiesis and anemia and included papers by Bethell (Ann Arbor) on PTAH, Daft (Bethesda), Experimental Anemias, Jacobson (Cambridge, England), Enzymatic Activation of Folic Acid, Tinsley and Moore (St. Louis), Inhibition of Erythropoiesis by High Oxygen Concentration, Finch (Boston), Iron Metabolism in Hemochromatosis, Shemin and London (New York), Porphyrins, Chevallier (Paris) Hemolytic Anemia.

On the third day, Jones (Buffalo) discussed phase microscopy and histochemical methods, Gibson and Vallee, Zinc and Carbonic Anhydrase, Thorell (Stockholm), Cytochemical Aspects of Blood Cell Development, Gonzalez-Guzman (Mexico), Ictero-Anemia, Farber (Boston) Aminopterin in Leukemia. This last paper evoked a great deal of discussion.

On the fourth day, iso-immunization was the chief topic of discussion, with papers by Houghton (Columbus), Mourant (London), Levine (Raritan), Reid (Dallas), van Loghem (Amsterdam), Young (Rochester, N. Y.), Race (London), Dacie (London), Robb Smith (Oxford), and Bessis (Paris).

Thirty-eight scientific exhibits on display throughout the sessions dealt with lymph node aspiration, marrow puncture, red cell abnormalities, folic acid antagonists, hemolytic transfusion reactions, sludged blood, multiple myeloma, phase microscopy, electron microscopy, P<sub>32</sub> therapy, spleen and hypersplenism, Rh factor, vitamin B<sub>12</sub>, etc.

The following officers were elected

*President* Professor Sir Lionel Whitby, Cambridge, England

*Vice-Presidents* For North America Dr. William Dameshek, Boston

For South America Dr. Luis Sandoval S., Santiago, Chile

For Europe Dr. G. DiGuglielmo, Naples, Italy

*Counsellors* (To be elected from each country) The three counsellors elected for the United States were as follows

Charles A. Doan, Columbus, Ohio

Edwin Osgood, Portland, Oregon

Ernest Witelsky, Buffalo, New York

The next congress of the Society will be held in Cambridge or Oxford, England in 1950.

In the Constitution, which was formally adopted, it was stated that the requirements for membership for those having the M.D. or Ph.D. degree include a sustained interest in some phase of hematology of at least five years standing.

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# BOOK REVIEWS

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*Il Principio Antianemico-Pernicioso* By G. ASTALDI AND M. BALDINI Monograph of *Il Farmaco*, January 1948 pp 234

This monograph offers a rather complete review of what has been accomplished and written on the subject of antipernicious anemia preparations since Minot and Murphy's fundamental discovery in 1926.

The main interest of the authors seems to be in the various methods for standardization and dosage of antianemic preparations. They find that no method so far suggested is completely satisfactory and propose a test of their own. This consists of studying the reduction in size of nucleated red cells as the transformation of megaloblasts into normoblasts takes place in the marrow of patients with pernicious anemia. Two marrow samples are obtained and studied, one before administration of the material to be tested and one on the sixth day after it has been administered. They feel that from a therapeutic standpoint the antimegaloblastic unit corresponds to the antianemic unit (U.S.P.).

The work is well up to date with a short chapter on folic acid and other products which have appeared recently. This part of the work could profitably have been more extensive.

DAVIDE LIMENTANI

*Cytologie Sanguine Normale et Pathologique* By M. BESSIS Paris, Masson & Cie, 1948 pp 298

With this work, Bessis offers the student of hematology in a pleasant and attractive fashion the old subject of morphologic hematology much too often cast aside today.

This achievement has been accomplished through a limitation of theoretic considerations and elimination of speculative and polemic discussions. The descriptive part of the text is maintained on a plane of reality and is clarified by the large number of black and white figures and by 19 colored plates. The illustrations, in the form of black and white half tones and of colored tables, are in general good and some are particularly fine.

The material is divided into ten chapters devoted to cytologic techniques, reticulo-endothelial system, quantitative study of the cells of blood and hemopoietic centers, stem cells, erythrocytic, granulocytic, thrombocytic, lymphocytic and histiocytic series and to the cells accidentally found on the smear of blood and hemopoietic organs. Each chapter includes a section on the morphology of pathologic changes involving the subject under discussion. Instructions for puncture of lymph nodes, spleen, liver, tumors, masses, etc., and information concerning normal and pathologic morphologic findings are given. The American reader may not care for the often involved European glossary.

This work does not include any information regarding phase microscopy and histochemical methods which are becoming so much a part of morphologic hematology. The book may be regarded as a valuable addition to the morphologic hematology library.

DAVIDE LIMENTANI

*Maladies du Sang et des Organes Hematopoesiques* By JEAN BERNARD Paris, Editions Medicales Flammarion, 1948

This beautifully printed and illustrated book of over 1000 pages represents one volume of a loose leaf system of medicine. There are many colored plates of blood and bone marrow cells. The various hemologic disorders are classified in a rather unusual fashion: those of acute type, those which are chronic (including pernicious anemia, polycythemia, chronic leukemias, etc.), those of constitutional or genotypic type, and those caused by various known factors such as infections and parasites. The whole work is carried out very simply and systematically, perhaps too systematically. There is very little stress placed upon fundamental physio-pathologic principles. The book is recommended for practitioners who wish to obtain a quick and authoritative review of a particular subject, systematically and lucidly presented.

WILLIAM DANESHEK

# BLOOD

## *The Journal of Hematology*

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### CYTOLOGY OF RABBIT THYMUS AND REGENERATION OF ITS THYMOCYTES AFTER IRRADIATION, WITH SOME NOTES ON THE HUMAN THYMUS

By HAL DOWNEY, PH D

*With the assistance of RUBY ENGSTROM, M A , M D*

IT IS now generally conceded that the "thymocytes" are lymphocytes having an embryonic origin in the mesenchyme surrounding the epithelial anlage of the thymus, as shown by Maximow,<sup>1 2</sup> Hammar,<sup>3-6</sup> Hartmann,<sup>7</sup> and others. A few investigators (Fulci,<sup>8</sup> Goldner,<sup>9</sup> Dustin,<sup>10</sup> Schaffer,<sup>11</sup> Stohr,<sup>12</sup> Pappenheimer,<sup>13, 14</sup> Winiwarter,<sup>15</sup> Gottesman<sup>16</sup> and Jaffe<sup>17 18</sup>) have derived the free thymus cells from the epithelium, believing that they are special parenchymatous elements of the organ and not lymphocytes, or they are genuine lymphocytes derived from the epithelium (Schaffer,<sup>11</sup> Prenant,<sup>19</sup> Gottesman<sup>16</sup> and Jaffe<sup>17 18</sup>). However, investigations of the embryonic development of the thymus, especially the detailed studies of Maximow<sup>1 2</sup> and Hartmann,<sup>7</sup> and experiments with radiation, transplantation, tissue culture, etc., (Dantschakoff,<sup>20</sup> Hammar,<sup>3-6</sup> Jolly,<sup>21</sup> Popoff<sup>22 23</sup>) have convinced most investigators that the thymocytes are true small, medium and large lymphocytes identical to those of the lymph nodes, and that, like the latter, they are derived from mesenchymatous tissue.

As the normal thymus does not have germinal centers and lymph nodules it can be assumed that most of the lymphocytes of the adult organ, the majority of which are small lymphocytes, are descendants of the large lymphocytes which invade the epithelial anlage from the mesenchyme of the embryo. (Hartmann<sup>7</sup> (1915) states that the immigration of these large, basophilic, ameboid lymphocytes begins at about the fifteenth or sixteenth day in the rabbit fetus, and that it subsides gradually. Transformation of these cells to small lymphocytes begins on the nineteenth fetal day, and all intermediate stages in the transformation are present between the nineteenth and twenty-fifth days, after the epithelial anlage has been completely penetrated by the large cells.)

Maximow<sup>1 2</sup> saw many of these first large lymphocytes in mitosis and concluded that the production of small cells was largely by division of the large ones. (Hartmann<sup>7</sup> could not determine the exact mechanism and seemed to assume that there

From the Department of Anatomy, Medical School, University of Minnesota, Minneapolis, Minn

is direct transformation of large to small cells. Many of the latter were in mitosis which, she thought, proved that they were not all derived from large lymphocytes.

To what extent the local stroma can contribute new lymphocytes, especially in the adult, is somewhat uncertain. The question is important from the standpoint of the maintenance of lymphocytes in the normal organ and their regeneration following pathologic involution. It was the main problem in the present investigation.

That the bulk of the supporting stroma is composed of an epithelial remnant of the embryonic anlage has been shown in detail by Hammar,<sup>3-6</sup> Maximow<sup>1, 2</sup> and Hartmann.<sup>7</sup> The epithelium becomes disorganized by the immigration and proliferation of lymphocytes and assumes a superficial resemblance to the reticulum of lymph nodes. Hart<sup>24</sup> (1912), Rudberg<sup>25</sup> (1907), Pappenheimer<sup>13</sup> (1910), Hammar,<sup>3-6</sup> Ssyssojew<sup>26</sup> (1924), Wituschinski<sup>27</sup> (1926), Marine<sup>28</sup> (1932) claim that this epithelial 'reticulum' can form histiocytes, macrophages and giant cells, but the conclusion of Schaffer<sup>11</sup> and of Gottesman<sup>16</sup> and Jaffe<sup>17, 18</sup> (1924) that this tissue can also produce genuine lymphocytes is denied by the above authors and most other students of the problem. Ssyssojew,<sup>26</sup> who studied accidental involution in children, concluded that the cells of the epithelial reticulum behave exactly like those of the reticulo-endothelial system, and that they should be included with this system in spite of their different origin.

The problem is difficult because mesenchymatous tissue becomes mixed with the epithelial portion and it is usually impossible to distinguish the two tissues in sections of the late fetal and adult stages.

Fibers, interpreted as connective tissue fibers, were seen in the thymus by Watney<sup>29</sup> (1882), Bell<sup>30</sup> (1906), Mietens<sup>31</sup> (1909), Pappenheimer<sup>13</sup> (1910), Salkind<sup>32</sup> (1912), Hartmann<sup>7</sup> (1915), Strandberg<sup>33</sup> (1918), and Jolly<sup>21</sup> (1923). Their presence except for those radiating from septa and blood vessels is denied by Badertscher<sup>34</sup> (1915). Strandberg,<sup>33</sup> using the Bielschowsky method, made an intensive study of the fibers in the human thymus from fetuses, newborn and adults up to the age of 54. He found that the black silver-staining fibers increased with age. They were most abundant in the medulla and the boundary between cortex and medulla. They radiated from the septa and from the blood vessels which penetrate the organ, and they penetrated the medulla for considerable distance beyond the vessels and septa. He could not determine relationship of fibers to cells and could not distinguish between epithelial cells and mesenchymatous reticular cells. The article is accompanied by excellent lithographed plates. The writer's preparations of rabbit thymus stained with azo-carmin show practically identical relationships of the blue-staining fibers to parenchyme as those described by Strandberg for the human thymus.

Mietens<sup>31</sup> (1909) had observed numerous fibers penetrating the epithelial reticulum of the medulla in human and animal thymus from deep ends of septa and from perivascular tissue. The fibers become embedded in this reticulum but they do not contribute to its formation. Numerous thin fibrils in the cellular reticulum were thought to be precollagenous, while the thicker ones derived from septa and perivascular tissue of the larger vessels, and stained blue with Mallory, are collagenous.

Jolly,<sup>21</sup> in 1923 and earlier, agreed that the thick fibers are collagenous. He could not stain the thin fibrils of the epithelium with Mallory's connective tissue stain, but could stain them with iron hematoxylin after prolonged mordanting, and so interpreted them as mitochondrial formations similar to those of the epidermis.

Investigations of others show that, mixed with the epithelial stroma there is a certain amount of mesenchymatous tissue which is undoubtedly responsible for the production of the reticular fibers. The problem was studied by Salkind<sup>32</sup> (1912), Mollier<sup>35</sup> (1913), and in great detail by Hartmann<sup>7</sup> (1915) who investigated the histogenesis of the rabbit thymus. Hartmann found that mesenchyme penetrates the embryonic anlage with the connective tissue septa and the blood vessels. At first it can be distinguished from the epithelium but later this distinction is impossible. As capillaries develop throughout the organ she assumed that they must be accompanied by mesenchyme which has the capacity to form new lymphocytes. During development of the thymus the narrow connective tissue septa contain numerous lymphoid cells and this tissue becomes a true lymphatic tissue, while the tissue surrounding the lobules remains as an ordinary connective tissue. She noted that the boundary line between the epithelium and the lymphatic tissue was soon obliterated and that the two tissues intermingled, a large portion of the septal tissue becoming a part of the organ parenchyme. She believed, but could not prove, that the mesenchymatous reticulum continues to form lymphocytes. One of the important conclusions from this work was, that the thymus stroma contains much more mesenchymatous tissue than generally had been assumed.

Salkind<sup>32</sup> stated that he could see lymphocytes budding from the mesenchymatous reticulum within the thymus of the adult.

Tschassownikow<sup>36</sup> found it impossible to distinguish the two tissues except in tissue culture, where they separate.

Popoff<sup>23</sup> (1928), a student of Maximow's, investigated rabbit thymus by the tissue culture method. He found that the epithelium formed islands and a border at the edge of the lobules. Some of its cells became isolated from the syncytium, but they never phagocytized or stored colloidal dye. Later the epithelium degenerated and lymphocytes, histiocytes or myelocytes were never formed from it. Histiocytes were numerous in the earliest cultures. They were derived from the connective tissue partitions between the lobules, and in the interior of the organ, from undifferentiated mesenchyme cells about the blood vessels. Undifferentiated mesenchyme was found everywhere in close association with the blood vessels, and it is this tissue which produces histiocytes and lymphocytes in the autotransplanted thymus and during regeneration following radiation.

Emmert<sup>37</sup> (1936) also experimented with tissue cultures of embryo beef and human thymus and of 3-7 week old rats. She found that the outgrowth of epithelial reticulum could be distinguished from that of the mesenchymatous reticulum. The cells of the latter were smaller than those of the former and their nuclei were different. The epithelial reticulum formed sheets of cells in the later cultures. In two or three days after subculture there was an outgrowth of epithelioid tissue which differed from the outgrowth of the other two tissues. Its narrower bands had a distinct margin composed of fibroblast-like cells. Later some of the epithelioid

tissue broke up into individual round cells. This tissue was assumed to be a remnant of the original epithelium which had not been transformed to reticulum. No proof was offered for this conclusion.

From the foregoing statements from some of the literature it can be asserted that the thymus does contain a considerable amount of mesenchymatous tissue mixed with the epithelial stroma and that it is probably this tissue which produces the fibers described by numerous authors. From the character of the fibrils and from its activity in the production of histiocytes and lymphocytes (Popoff<sup>22-23</sup>) it can be concluded that this tissue is similar to the reticulum of lymph nodes, or that it is an undifferentiated mesenchyme closely associated with the vessels, as claimed by Popoff.

The writer was interested in determining whether the mesenchymatous reticulum can be distinguished from the epithelium in imprints or smears of the organ, and, if it can, to what extent it contributes to the formation of new lymphocytes in the normal organ and during regeneration following irradiation. He was also interested in studying the thymus lymphocytes in imprints, an interest which was stimulated by Dr. Arthur Kirschbaum, who showed him some very immature lymphocytes in imprints of the normal mouse thymus.

Pinner<sup>28</sup> (1915), a student of Pappenheimer's, seems to be the only one who has made a detailed cytological study of the thymus by the smear method. His interest was focused largely on the question of whether the small thymus cells are lymphocytes or epithelial derivatives.

The origin of myelocytes and mature granulocytes of the thymus was another problem studied in the present investigation. Their presence in the thymus has been described many times but there is no agreement on their origin. Some, like Hart<sup>4</sup> and Fulci,<sup>8</sup> believe that they immigrate from the blood or connective tissue. Hart saw scattered eosinophil myelocytes in the human thymus, but believed they were the result of special conditions rather than an indication of hematopoiesis. He thought the blood was the most likely source. Wituschinski<sup>27</sup> (1926), experimenting with the introduction of foreign bodies in the thymus, derived them from adventitial cells of the vessels and from "hemocytoblasts," some of which originated from the epithelial stroma.

The prevailing theory for the origin of the thymus myelocytes is that they are derived from the lymphocytes of the organ. Maximow<sup>1</sup> (1909) described the development of myelocytes from large lymphocytes of the thymus of fetal rabbit and other animals. Danchakoff<sup>20</sup> (1916) showed a similar origin in the thymus of chick embryos after implantation of spleen grafts on their allantois.

Weidenreich and his student Weill made a detailed study of this problem. The main features were published by Weidenreich<sup>39</sup> (1912) and the details by Weill<sup>40</sup> (1913). Their material was taken from full grown rats and from 4 normal human subjects, aged 15, 17, 19, and 37 years. The 19 and 37 year olds were executed and the material was placed in the fixing fluid immediately afterwards. The other 2 were cases of accidental deaths.

The rat thymus was valuable because of the ring-shaped nucleus in the hetero-

phils and eosinophils which facilitated the study of the development of these cells from lymphocytes. All transitional stages in the process could be seen. The thymus cells also developed to plasma cells. The basophils were of the tissue type and were located in the connective tissue only.

The thymus from all 4 human subjects contained neutrophil and eosinophil myelocytes. Eosinophils were seen in every section, and even in the 37 year old subject about 35 per cent of them were myelocytes. The nuclei of the myelocytes varied from large leptochromatic to small pachychromatic nuclei similar to those of the small cells. Many myelocytes were in mitosis. Transitions between lymphocytes and plasma cells were seen also in the human thymus, and a few plasma mast cells were noted. Other mast cells were of the tissue type.

The authors concluded that the free round cells of the thymus are genuine lymphocytes of various sizes similar to those of lymph nodes. All types of lymphocytes, even the small ones, can develop to granulocytes and plasma cells within the thymus, and this is a normal process at all ages.

The authors did not mention the possibility of the development of granulocytes from mesenchymatous tissue located within the limits of the thymus parenchyme.

Pinner<sup>38</sup> made smears from the human thymus of 25 cases ranging in age from newborn to one year, and from one 8 week old rabbit. Two types of myelocytes were seen. One type had large, pale nuclei and was identical to the marrow myelocytes, the other type had small, dark lymphocytic nuclei. Both forms occurred in the promyelocyte stages with few granules. Pinner assumed that they were derived from lymphocytes, but he did not give any of the details.

Hartmann<sup>7</sup> saw some myelocytes with nuclear structure similar to that of large lymphocytes in sections of rabbit thymus. She derived the granulocytes of the organ from large lymphocytes, but was not certain that they were of local origin, because she could not find eosinophil myelocytes containing only a few granules and because the neighboring connective tissue contained many granulocytes.

Ssyssojew<sup>26</sup> saw extensive myeloid metaplasia during pathologic involution in 15 children who had died of infectious diseases. The neutrophil promyelocytes were identical to the large lymphocytes except for the presence of granules in their cytoplasm. There was also myeloid metaplasia in the surrounding connective tissue, and in nearby vessels which had its beginning with large lymphocytes. Neutrophils were the most numerous granulocytes.

Popoff<sup>22</sup> (1926), in regenerating stumps remaining after partial extirpation and of transplants, noted the formation of lymphocytes of different sizes from the connective tissue which invaded the hypertrophied epithelium with the vessels. Some of these lymphocytes developed to myelocytes which were mostly of the darkly nucleated, micromyelocytic variety.

Fulci<sup>8</sup> (1913) stated that the thymocytes are not lymphocytes because they do not differentiate to plasma cells or granulocytes. However, the foregoing extracts from the literature indicate that there is good evidence for the origin of granulocytes from these cells, and Schaffer,<sup>11</sup> Weidenreich<sup>39</sup> and Dantschakoff<sup>20</sup> showed that they can form plasma cells. As the material studied by the writer contained many



myelocytes in both sections and imprints it was felt that renewed study of this question might be of interest. The imprints seemed to be of special importance because they showed the finest details of nuclear structure, which is a valuable feature in determining the origin of cells.

### MATERIALS AND METHODS

The animals used were 10 newly born rabbits and 2 young normal rabbits, age 4 or 5 months. One young rabbit, aged 4 or 5 months, was irradiated with a dosage of 500 r u. This animal was killed on the sixth day after irradiation. Its thymus was large. One old rabbit was given 400 r u and killed eight days after irradiation. Its thymus was in an advanced stage of involution and showed epithelial hyperplasia. One old normal rabbit was not irradiated.

Some human material was also used for imprints. The specimens were from 2 full term newborns, 2 stillborns who died shortly before birth, one 2 year old male, one 5½ months fetus, and one premature aged 6½ fetal months.

Sections and imprints were made from all of these animals and imprints from the human material. Sections were stained with hematoxylin and eosin, Dominici's eosin-orange G-toluidin blue, and with Pappenheim's methyl green and pyronin. A few were stained with azocarmine for study of the distribution of connective tissue fibers. Sections stained with methyl green and pyronin were dehydrated with dioxan, as suggested by Miss Helen Harris of this laboratory. Retention of stain was much better than with the usual acetone or alcohol dehydration.

The imprints were made by touching a cut surface of the organ to a slide without smearing. The preparations were dried rapidly by waving them through the air, rapid drying being essential for good films. All imprints were stained with the May-Grunwald Giemsa combination. The Giemsa stain was used in double strength, 6 drops of stock solution in 3 cc. of distilled water for each slide for five or six minutes. The May-Grunwald solution was used like Wright's stain, one minute in the stock solution and three minutes after the addition of water. The diluted stain was poured off and the Giemsa added without rinsing. The slides were not covered because it was intended to use only the oil immersion objective. Most of the imprints showed some portions that were good for cytological study.

### OBSERVATIONS (RABBIT)

The irradiated animals were used for the study of regeneration of lymphocytes in the thymus, the others for detailed study of the lymphocytes and of cells from the epithelial and mesenchymatous reticulum and for the granulocytes.

There was little histologic or cytologic difference between the thymuses of the newborn animals and those from the young adults, aged 4 or 5 months. In the newborn there were, however, fewer large lymphocytes and, in the imprints, more epithelial cells than were seen in the young adults. The presence of more epithelial reticular cells in the imprints of the newborn probably was due to the delicate texture of the organ which caused more of the epithelial cells to adhere to the glass.

### EPITHELIAL AND MESENCHYMATOUS RETICULUM

In the imprints, the mesenchymatous cells usually could be distinguished from those of the epithelium, as shown in figures 5, 6 and 7 from newly born rabbits. Figures 5 and 6 are epithelial cells and figure 7 is a mesenchymatous cell identical to the reticular cells of lymph node imprints. The epithelial cells have rather sharp outlines when they are free and their cytoplasm is of a translucent pale blue color when stained with May-Giemsa. It is not completely homogeneous, for some areas

are slightly more basophilic than others, and it usually contains many small vacuoles seen best in the darker areas. It does not have the larger vacuoles usually present in the mesenchymatous cells or inclusions of phagocytized material in the normal animals.

Figures 5 and 6 illustrate variations in structure of the nuclei of the epithelial cells of the imprints. As seen in figure 5, the chromatin has a rather uniform distribution in the form of a network of broad strands which do not stain very intensely. This network may become more sharply defined and there may be some clumping of chromatin, as seen in figure 6. Nucleoli were not seen in the imprints, but in sections many of the epithelial cell nuclei contain a sharply defined spherical nucleolus which, with Dominici's stain, is colored light pink while the chromatin is blue. Pinner<sup>38</sup> saw nucleoli in some of the epithelial cells of imprints of human thymus. The nuclear membrane is extremely thin and poorly defined.

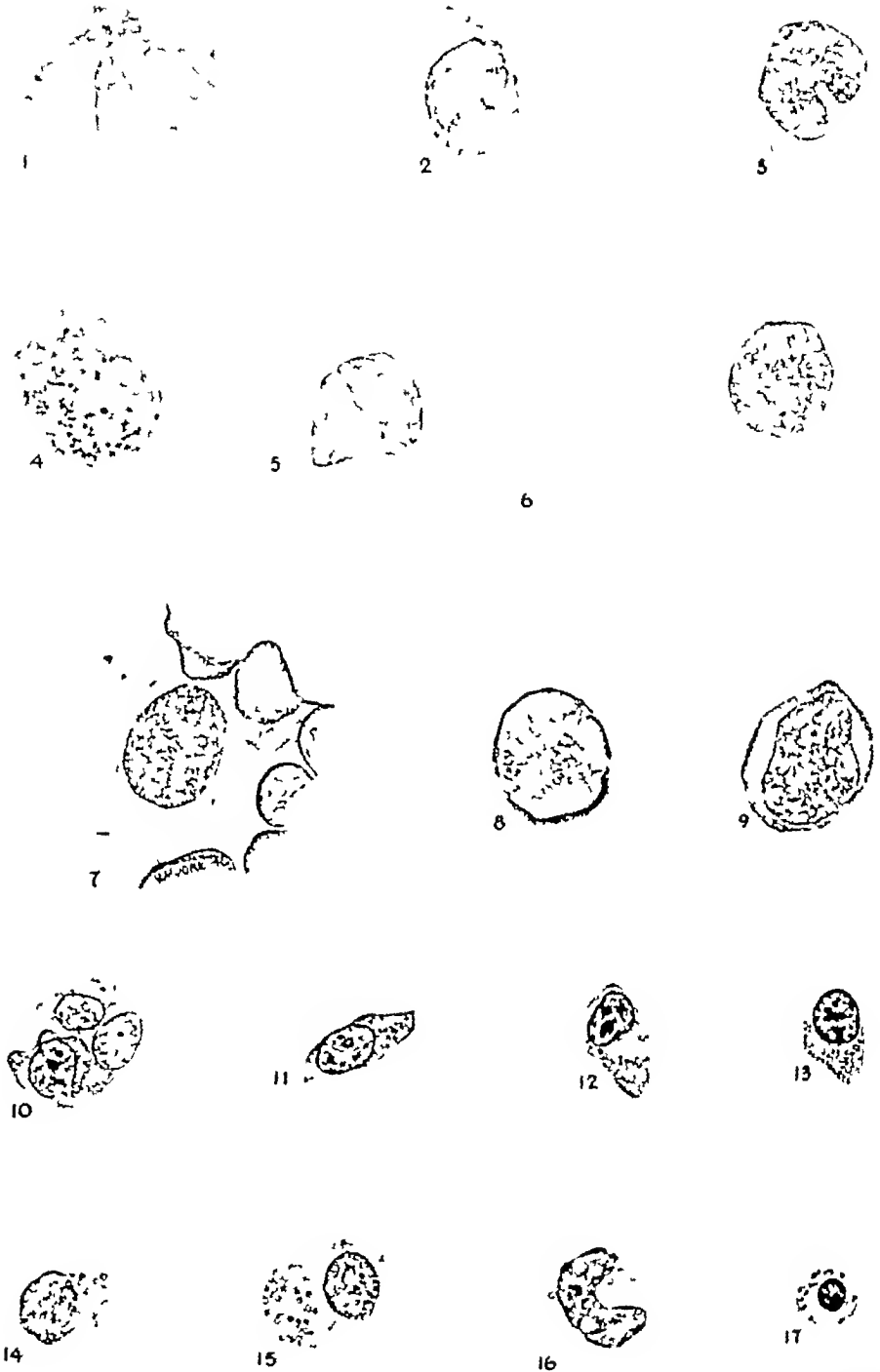
In the imprints the nucleus is round (fig. 6) or slightly irregular in shape, and in many of the cells it has a deep groove on the surface (fig. 5). These nuclear grooves also are seen in the sections, especially in the inner portion of the cortex and in the medulla, where the shape of the nucleus tends to be more irregular than in the outer portion of the cortex. In the latter region the nuclei are usually round or elliptical. In the sections the chromatin is condensed to a few scattered small granules which do not condense around the nucleoli. This results in an extremely pale nucleus with any of the stains used. The cytoplasm also shows very little affinity for any stain.

Cells like those of figures 5 and 6 are not numerous in the imprints, probably because they are not easily detached from their fixed position. They are most numerous in the imprints from the newborn animals. Many of them are damaged in the imprinting but usually can be identified by their peculiar cytoplasm in case the nuclear damage is too extensive.

The imprints do not have many histiocytic reticular cells like the one of figure 7 but there are enough of them to indicate that they are an important constituent of the stroma. They are identical to the reticular cells of imprints of lymph nodes illustrated by Sundberg and Downey<sup>41</sup> (1942) in their figure 7, and in figure 4 of Downey and Stasney<sup>42</sup> (1936). They are not fibrocytes of the septa or surrounding connective tissue because fibrocytes do not show the pronounced stippling of the nuclear chromatin of reticular cells and their cytoplasm is more homogeneous.

The reticular cell of figure 7, and others like it, differs in both nucleus and cytoplasm from the epithelial cells of figures 5 and 6. The cytoplasm has a pinkish cast due to the presence of numerous small azurophilic granules. It usually contains several large vacuoles, often with inclusions of phagocytized material. It seems to be more fluid in texture than that of the epithelial cells, for it usually extends for some distance beyond the nucleus and flows between neighboring lymphocytes, as seen in figure 7.

The nucleus stains darker than that of the epithelial cells because the chromatin is condensed in the form of small granules which gives the nucleus a stippled appearance. There are some open spaces in which the violet parachromatin is conspicuous. There may be some clumping of the chromatin granules to form chroma-



† All figures are from thymus of rabbit. All were painted with the same magnification, Leitz  $\frac{1}{4}$  oil immersion objective and ocular  $10\times$ . Camera lucida outlines. The imprints were stained with May Grünwald Giemsa and the sections with Dominici's eosin orange G toluidin blue.

FIGS. 1, 2, 3. Pseudoeosinophil promyelocytes and metamyelocyte. Imprint from 5 day-old rabbit. In fig. 1 the nucleus shows some stippling of chromatin that is characteristic of cells of the mesenchymatous reticulum, like the one of fig. 7, suggesting origin of granulocytes from this reticulum. Such an origin as seen in sections is illustrated in fig. 10.

tin blocks. The beginning of this process is shown in figure 7. The type of stippled nucleus shown in figure 7 is characteristic of reticular cells regardless of their location. Free histiocytes also show this same type of nucleus even when they are derived from lymphocytes, as is often seen in inflammation of the loose connective tissue, as shown by Kolouch<sup>43</sup> (1939) in his figure 7b.

In imprints it is sometimes difficult to distinguish cells of the epithelial reticulum from those of mesenchymatous origin. The epithelial cells may round up and become free and there may be considerable clumping of chromatin. Usually the sharp nuclear groove or the pale translucent cytoplasm will serve to identify them as epithelial cells. However, where the cells are crowded between lymphocytes it may be impossible to classify them, especially when the mesenchymatous cells are undifferentiated and do not have the histiocytic type of cytoplasm seen in figure 7. In sections the difficulty is, of course, still greater.

Pinner,<sup>38</sup> who studied imprints of human thymus, does not mention the presence of any mesenchymatous cells. He did see epithelial cells with very leptochromatic nuclei some of which had nucleoli. They were not described or illustrated in detail.

In the sections the mesenchymatous tissue often can be identified in some areas under the capsule and around the blood vessels which penetrate the cortex and medulla. When stained with azocarmine blue connective tissue fibers are seen to be most numerous in the medulla around the blood vessels but not entirely confined to the immediate neighborhood of the vessels. Some very thin fibers radiate for some distance from the vessels into the parenchyme, and some thin fibers penetrate

FIG. 4. Promyelocyte of mast leukocyte. The nucleus is similar to the one of fig. 1. Same imprint as figs. 1-3.

FIGS. 5 and 6. Epithelial reticulum cells from imprint. Note nuclear groove in fig. 5 and clumping of chromatin in fig. 6. The cytoplasm is characteristic and unlike that of the mesenchymatous cell of fig. 7. Same imprint as figs. 1-4.

FIG. 7. Imprint of cell from mesenchymatous reticulum. Newborn. Compare nucleus and cytoplasm with the epithelial cells of figs. 5 and 6. Note characteristic stippled nucleus and azurophilic granules in cytoplasm of fig. 7.

FIGS. 8 and 9. From imprint of newborn. FIG. 8. Lymphoblast showing very diffuse distribution of chromatin. In fig. 9 there is some clumping of chromatin and some stippling suggesting origin of this cell from mesenchyme.

FIG. 10. Section, young normal adult. Development of heterophils from mesenchymatous reticulum under capsule. Nucleus of upper granulocyte similar to nucleus of mesenchyme. Cytoplasm of mesenchyme cell not shown because it is unstained until granules begin to develop. The lower granulocyte shows that chromatin increases as the cytoplasm fills with granules.

FIGS. 11 and 12. Section, young normal adult. Hemocytoblastic myelocytes in septum. A slight amount of basophilic cytoplasm is still present.

FIG. 13. Section, young adult. Heterophil myelocyte in cortex.

FIGS. 14 and 15. Imprint, young adult. Lymphocytic heterophil promyelocyte and myelocyte with nuclei of small lymphocytes. Compare these nuclei with those of figs. 1, 2, and 4.

FIG. 16. Imprint, young adult. Heterophil metamyelocyte which seems to have developed from a cell like 15.

FIG. 17. Section. Eosinophil myelocyte with nucleus of small lymphocyte. This cell was one of a group of 5 similar cells in interlobular connective tissue. Eosinophils of this type and transitions between them and the bilobed form occur in the medulla. All the eosinophils of the rabbit thymus seem to develop from small lymphocytes. FIGS. 14 and 15 prove that heterophils also may develop from small lymphocytes.

the cortex from the surrounding connective tissue Strandberg<sup>33</sup>(1918) illustrated the distribution of these fibers which stain black with the Bielschowsky technic They also have been described by others, so the details will not be given here On account of the black staining with Bielschowsky it may be assumed that the thinner ones are related to reticular fibers and that some of the cells that produce them are reticular cells similar to the one illustrated in figure 7

In sections the cytoplasm of these cells is almost colorless while they are in the inactive state Their nuclei are round or elliptical They contain little chromatin, but more and in coarser particles than is true of the epithelial nuclei They do not have nucleoli One of these nuclei is shown on the right of the 2 granulocytes of figure 10 The nuclear membrane is thicker than that of the epithelial nuclei The cytoplasm was not stained That cells of this type are mesenchymatous in origin is proved by the fact that some of them develop to granulocytes as illustrated in this figure This will be described in detail later A similar transformation of cells from the epithelial reticulum was not seen

#### LYMPHOCYTES

The lymphocytes of the imprints are of the small, medium-sized and large types seen in imprints of rabbit lymph nodes However, the nodes have some lymphocytes that are much larger than any encountered in the thymus imprints The cytoplasm of these cells is usually smooth, homogeneous except for a few small vacuoles, and very basophilic The nuclei are often immature in the sense that they show some characteristics of the nuclei of the reticular cells from which they are derived These large cells and the medium-sized lymphocytes have more cytoplasm than is seen about the nuclei of the lymphocytes of the thymus, and the nodes have many more extremely basophilic cells of all types Extremely basophilic cells are present in the thymus, but on account of the narrow rim of cytoplasm they are not very conspicuous The nodes also contain many more immature lymphocytes than are present in the thymus A detailed description of the lymphocytes of the lymph nodes and their regeneration will be found in the paper by Sundberg and Downey<sup>11</sup> (1942)

Small lymphocytes with very dark pachychromatic nuclei are the most numerous They have so little cytoplasm that often it cannot be seen The chromatin blocks are very dense and stain very dark blue which is almost black Slightly less numerous are larger small lymphocytes with about the same nuclear structure but with chromatin less dense and staining violet rather than blue black

Komocki<sup>44</sup> (1930) described and illustrated the dense nuclei of the small lymphocytes of the thymus He believed that the smallest cells have no cytoplasm Policard, Dustin and Marcus were quoted as supporting this opinion He believed that the nuclei of the smallest lymphocytes of lymph nodes have a different structure and that they always are surrounded by cytoplasm He agreed with Schridde that the nuclei of the small thymocytes are more loosely constructed than are those from the corresponding cells of the nodes His illustrations of the small thymocyte nuclei are accurate but the figures of lymphocyte nuclei from the lymph of lymph nodes do not in any way resemble the nuclei of lymphocytes of either sections or

imprints of nodes Pappenheimer<sup>13</sup> (1910) also believed that most of the small thymocytes have no cytoplasm that can be detected in smears, in which respect they differ from lymphocytes of lymph node smears. The thymocytes were thought to be epithelial elements.

It is true that most of the small thymocytes have very dark checker-board nuclei with sharply demarcated chromatin blocks separated by rather broad pale interspaces. Many of these nuclei are suggestive of the type seen in small plasma cells. Similar cells occur in lymph nodes of the same animals but they are not as numerous and their chromatin blocks usually are not as darkly stained. Naked nuclei similar to those of the thymus also occur in the nodes. Lymphocytes that are slightly larger than the smallest ones usually have cytoplasm in both nodes and thymus.

The writer concludes that every type of lymphocyte of the thymus can be duplicated in the nodes. However, certain types predominate in each of these organs, as has been shown in the preceding discussion.

Next in number in the thymus are medium and small large lymphocytes, usually with the nuclear structure of mature lymphocytes. Cells corresponding to the largest lymphocytes of the nodes do not occur. The cytoplasm of these thymus cells forms a narrow band about the nucleus, it is basophilic but usually not as dense and homogeneous as in the nodes. It usually has a light area at one side of the nucleus. Lymphocytes with abundant cytoplasm are scarce.

In sections narrow-bodied large lymphocytes are scattered among the small and medium ones of the cortex. There are very few large or medium-sized ones in the medulla. The largest ones with the most abundant cytoplasm tend to collect under the condensed connective tissue at the surface of the lobules. Here they approach in size the large lymphocytes of the nodes, although the nodes have some lymphocytes larger than any that occur in the thymus. Blood vessels of the medulla sometimes are surrounded by a dense collar of small and medium lymphocytes, a condition which seems to be more frequent during regeneration after radiation.

In the thymus imprints there is a fair number of large lymphocytes with very immature nuclei. In some of these cells it is diffusely distributed in the form of a delicate network or fine stippling without any clumping of chromatin. One such cell is shown in figure 8. Cells of this type are sufficiently immature to be called lymphoblasts. They resemble the lymphocytes of acute leukemia more than they do the immature reticular lymphocytes of the rabbit nodes. These blast cells are not numerous. Cells like the one of figure 9, in which there is some clumping of chromatin, are more numerous. The nucleus of this cell has some characteristics suggesting origin of the cell from a mesenchymatous reticular cell with a nucleus similar to that of figure 7. There is some clumping of chromatin but there is also some coarse stippling of the chromatin like that of figure 7. Some transitional forms between 7 and 9 could be found but they were not nearly as numerous as in lymph nodes. Illustrations of these transitional stages in lymph nodes are to be found in the papers of Downey and Stasney<sup>2</sup> (1936) and Sundberg and Downey (1942).

The presence of these transitional stages suggests that there is some development of lymphocytes from mesenchymatous reticulum in the thymus of normal animals. In the sections of normal animals it was impossible to see this line of development. However, during regeneration after irradiation of the thymus, as seen in sections stained with methyl green and pyronin, development of lymphocytes from the fixed tissue seems to be increased. This is probably the best stain that can be used for study of this process. The transition from fixed cells to free lymphoid cells was noted in several places at the periphery of the lobules near the capsule, and in the medulla close to the larger blood vessels. Downey and Weidenreich<sup>45</sup> (1912) published descriptions and colored illustrations of the development of lymphocytes from reticulum in sections of lymph nodes stained with methyl green and pyronin. The process is similar to what was seen in the irradiated thymus.

From the character of the intermediate cells of the imprints it seems clear that the fixed cells involved in this process are not of epithelial origin as some authors have assumed. Hartmann,<sup>7</sup> better than any other author, has given an explanation of the presence of mesenchymatous tissue in portions of the cortex and in the medulla in the neighborhood of blood vessels. The mesenchyme which penetrates the organ in early embryonic stages is undoubtedly the fixed tissue which is capable of forming lymphocytes under certain conditions. Hartmann believed this but she was unable to demonstrate it with the methods she used.

The presence in the imprints of reticular lymphocytes similar to those of the nodes can be explained by the activity of the mesenchymatous tissue in the production of lymphocytes. However, this does not account for the immature lymphocytes of the "blast" type similar to those of lymphatic leukemia. Naegeli<sup>65</sup> believed that the lymphoblasts were not "blasts" but lymphocytes which were about to divide by mitosis or had just completed a division. This might be an explanation for the presence of such cells in the thymus but it does not account for their absence in lymph nodes. Mitoses were not very numerous in the normal thymus material used in this study. Their number was increased during regeneration following irradiation and the "blast" cells also seemed to be more numerous. The lymphoblasts do not seem to be an intermediate stage in the development of lymphocytes from the mesenchymatous reticulum. However, the thymus is not very favorable material for study of this question because the lymphoblasts are so few in number. The development of similar cells from the reticulum has been seen in human lymphatic leukemia by Fineman<sup>46</sup> (1922) and by Stasney and Downey<sup>47</sup> (1935).

#### GRANULOCYTES

As noted in the introduction, many investigators have seen myelocytes in the normal animal and human thymus. Some, like Hart,<sup>24</sup> thought they resulted from special conditions and so were not to be interpreted as a sign of hematopoiesis. Others believed they were formed in the connective tissue surrounding the thymus or were brought in by the blood. Pappenheimer<sup>13, 14</sup> interpreted the eosinophil myelocytes as specifically differentiated epithelial elements which might have a secretory function. Hartmann<sup>7</sup> could not find eosinophil myelocytes containing

only a few granules so concluded they were developed outside the thymus, while the heterophils were of local origin. Weidenreich<sup>11</sup> and Weill<sup>10</sup> are among the few who have studied the granulocytes in sufficient detail to trace their origin and transformation to mature leukocytes.

In the imprints there are two types of promyelocytes in the heterophil series, viz., a type similar to those of the bone marrow (figs. 1-3), and the lymphocytic type (figs. 14-16) described by Weidenreich,<sup>11</sup> Weill<sup>10</sup> and Tuve.<sup>15</sup> In the youngest promyelocytes of the bone marrow type granules are not basophilic as they often are in the marrow. The basophil myelocytes (mast myelocytes) that were seen (fig. 4) were of the bone marrow type and were similar to the cells of the heterophil series (polymorphonuclears) except for the color and size of the granules. Eosinophil myelocytes and leukocytes were not numerous in the imprints. The nuclei of the myelocytes were of the small lymphocytic type, as shown in figure 17 which, however, is from a section. Imprinting does not change the morphology of these cells to any great extent.

The cells illustrated in figures 1-4 were compared to similar cells in excellent rabbit bone marrow smears loaned by Dr. Dorothy Sundberg of this Department. The lymphocytic type of myelocyte (figs. 14-16) was not seen in the marrow but cells comparable to those of figures 1-3 were numerous. However, there were some differences in structural details between the two series. The heterophil myelocytes of the thymus (figs. 1, 2) tend to spread out more and assume more irregular outlines than is true of the corresponding marrow myelocytes. This probably is due to difference in the character of the tissues. Owing to the thin spreading of the cytoplasm the granules of the thymus cells appear larger than those of the marrow.

In many of the marrow heterophil myelocytes some of the granules are basophilic, or of intermediate tints between basophilic and acidophilic. In the thymus the granules are always bright red. The only basophilic granules seen were in the mast leukocytes and their myelocytes (fig. 4). The coarse, dark 'myeloid azure' granules of irregular shape and size which are present in many of the marrow promyelocytes were not seen in the thymus imprints.

Nuclear structures of the myelocytes from the two tissues were quite similar. The nuclear pattern of the earliest promyelocytes is quite diffuse. It becomes coarser as the cells mature, as seen in figures 1-3. The nucleus of figure 1 shows some chromatin blocks which are not as dense as those of the more mature cells, and it also shows some stippling of the chromatin. In the marrow some of the promyelocytes have a more leptochromatic myeloblastic type of nucleus than those of the thymus. This may be only an apparent difference due to the greater number of myelocytes in the marrow which facilitates the finding of the various developmental stages.

The lymphocytic type of heterophil myelocyte seen in the imprints and illustrated in figures 14-16 was not seen in the marrow. The granules are identical to those of the marrow type of cell (figs. 1-3) but the nuclei are quite different, and the cytoplasm never shows more than a trace of basophilia. The nuclei are identical to those of small or medium lymphocytes. Their chromatin is in dense



blocks separated by clear interspaces and it stained very dark blue (fig 15) or violet as in figure 14. This difference in color probably is due to variations in the degree of spreading of the cells and their nuclei. The nucleus may be in the center of the cell but usually it is eccentric (figs 14, 15). A cell of this type with a central nucleus as seen in section is shown in figure 17.

In the marrow type of promyelocyte the cytoplasm is quite basophilic when the first granules appear. In the lymphocytic type the small lymphocytes develop a medium or wide cell body which becomes almost colorless. The first granules appear as very small pink specks in this colorless cytoplasm. A few of these cells with the nuclei of small lymphocytes (figs 14, 15) have a slightly basophilic area with few granules and a colorless area in which most of the granules are concentrated (fig 14). Cells of this type are not very numerous. That they complete their development is indicated by cell 16 which seems to be a metamyelocyte of this series. Several pseudoeosinophils with this type of nucleus, and transitional stages between this and the lobulated nucleus of the mature heterophil were found in the imprints.

In the sections most of the heterophil (polymorphonuclear) myelocytes are of the hemocytoblastic type of figures 11-13. Their nuclei are identical to those of the large basophilic lymphocytes of the sections. Their nuclear membrane is thick, the chromatin blocks are coarse, and there is good staining of the karyoplasm. Remnants of basophilic cytoplasm are often present (figs 11, 12), and in the younger promyelocytes it is obvious that the granules develop in a basophilic cytoplasm. A few heterophil myelocytes with dark, compact nuclei corresponding to those of figures 14 and 15 of the imprints were also seen in the sections.

All transitional stages in their development from large basophilic lymphocytes to mature cells can be found. Development of granules does not begin until the lymphocytes have acquired more than the usual amount of basophilic cytoplasm. Cells that are in compact groups are often in the same stage of development, thus, there may be several metamyelocytes in one group and only myelocytes in a neighboring group.

The distribution of these cells is quite irregular, they are numerous in some lobules, particularly the smaller ones that project for some distance into the surrounding connective tissue. Other lobules contain none. They are most numerous in the outer portion of the cortex close to the capsule, but they can also be located in any part of the organ. They are either scattered or in compact groups, and may be numerous in the medulla in the neighborhood of large blood vessels and connective tissue strands.

Another type of pseudoeosinophil myelocyte which usually occurs in groups of 2 or 3 cells immediately under the capsule is seen occasionally. Two such cells are shown in figure 10. Their nuclear membranes are thinner and the nuclei are paler than those of the hemocytoblastic type due to the lighter staining of the karyoplasm and the smaller and more diffusely distributed chromatin granules. The cytoplasm of the younger promyelocytes with few granules is only slightly basophilic, as seen in the upper cell of figure 10. The basophilia increases as the cells acquire more granules (lower cell of fig 10).

These cells develop from the stroma under the capsule. This reticular stroma is probably of mesenchymatous origin. Its nuclei have slightly thicker membranes, more chromatin, and absence of nucleoli to distinguish them from the nuclei of the epithelial reticulum. One such nucleus is shown on the right of figure 10. The cytoplasm could be seen but was practically colorless so has not been included in the figure. The nucleus of the upper myelocyte is identical to this nucleus, although somewhat smaller. The nucleus of the lower myelocyte has more chromatin and slightly darker karyoplasm, but it is not the lymphocytic type of nucleus of figures 11-13. It is possible that such a cell may eventually resemble the lymphocytic type. This point could not be settled because there are so few myelocytes developing from the fixed tissue. Some cells similar to the upper one of figure 10 were seen in the medulla where there may be much mesenchymatous tissue. It is logical to assume that they also have originated from mesenchymatous reticulum.

The rabbit is an animal that has few eosinophil leukocytes in its blood, marrow and thymus. While the total number in the thymus seems to be small they may be numerous in the medulla of some lobules. Their development in rabbit marrow from myeloblasts was described by Ringold<sup>49</sup> (1921) who showed marked changes in size and staining reaction of the granules as they matured. These changes in staining reaction of the granules were not seen in the imprints or sections of the thymus. The granules are larger and not as bright red as the pseudo-eosinophil granules and they are more refractile. They are often spindle shaped in the sections. Only a few of the mononuclear eosinophils had cytoplasm that was not completely filled with granules, which sometimes were a little smaller than those of the more mature cells.

Small and medium lymphocytes with dark checker-board nuclei seem to be the progenitors of all the eosinophils which are developed in the thymus and of a few of the marrow eosinophils. When the nucleus becomes bilobed the chromatin pattern remains essentially that of the small lymphocyte, although there may be some condensation of chromatin to form the cart-wheel type of nucleus. Tuve<sup>48</sup> and Weill<sup>49</sup> described two types of eosinophil myelocytes in human thymus, those with large leptochromatic nuclei and those with the nuclei of small lymphocytes. Only the latter type could be found in the rabbit.

Mononuclear eosinophils like the one of figure 17 often occur in groups in the interlobular connective tissue and in the septa. The cell of figure 17 is one of a group of five similar cells. Because of the number of these cells in the group, and because similar cells were seen in the imprints, one cannot assume that the nucleus is merely a section through one lobe of a bilobed nucleus, as was claimed by Schridde<sup>50</sup> for similar cells. Single cells of the same type are found occasionally in the medulla, and in some portions of the medulla, near large vessels and connective tissue strands, they and mature eosinophils with bilobed nuclei are quite numerous.

Mature mast leukocytes (basophils) with lobulated nuclei and a few of their promyelocytes and myelocytes were seen in the imprints of the thymus. They could not be identified in the sections, probably because the rabbit mast granules are very soluble in water. One of the promyelocytes is illustrated in figure 4. The granules have a violet or purple color with May-Giemsa staining and they vary somewhat

in size and shape. The nucleus of cell 4 is typical of the other basophil myelocytes that were seen. Its structure is identical to that of the bone marrow myelocytes whose origin could be traced to the myeloblasts. In the marrow smears examined the cells are not spread as thin as the one of figure 4, which probably accounts for the smaller size and darker staining of the granules of the marrow cells. Tissue basophils are very scarce in the rabbit and none was seen in the thymus of this animal.

The origin of some of the granulocytes of the thymus is not clear, and comparison of granulocytes of sections with those of the imprints is sometimes difficult. The cells of figures 1 to 3 seem to belong to one series. If one saw only cells 2 and 3 he would be inclined to believe that they were derived from lymphocytes on account of their nuclear structure. Cell 1 has a more immature nucleus with some stippling of the chromatin and some rather pale chromatin blocks which are composed of small chromatin granules. A nucleus of this type could have originated from a reticular nucleus, like that of figure 7, or from the nuclei of immature lymphocytes (figs 8, 9) which in turn may have been derived from the mesenchymatous reticulum. Cells 2 and 3 show that the nucleus acquires a coarser structure as the cells mature. This corresponds to the maturation of similar cells in the marrow and so does not necessarily mean that the cells have been derived from lymphocytes.

In myeloid metaplasia lymphocytes may change the structure of their nuclei to the type that is characteristic of the myeloblast before the cells develop granules. This dedifferentiation of the cells was noted especially by Dominici<sup>51</sup> (1921 and earlier) and by Maximow<sup>2</sup> (1923). Dominici<sup>51</sup> studied experimental myeloid metaplasia of the spleen and noted that some of the lymphocytes passed through a 'myeloid' stage during their evolution to the granulocyte while others did not. In the myeloid type of evolution the lymphocytes assume the character of myeloblasts with pale nuclei and little chromatin. In the lymphatic type of evolution lymphocytes of any type acquire granules without any further changes in nucleus and cytoplasm. In both cases the cells enlarge before producing granules, but in the myeloid type the nucleus enlarges more than the cytoplasm, so that the cytoplasm becomes relatively narrow and very basophilic. The lymphatic type of evolution is the more common one in the normal animal.

Maximow<sup>2</sup> (1923) saw myeloid metaplasia in cultures of rabbit lymph node to which cell free bone marrow extract had been added. All of the granulocytes developed from lymphocytes. In some instances the lymphocytes acquired large pale nuclei before developing granules, in others the lymphocytes were practically unchanged when they developed their granules. These observations are identical to those of Dominici<sup>51</sup> in the mammalian spleen.

The dedifferentiation of lymphocytes which may occur before they develop granules makes it difficult to determine in all cases the origin of cells such as those illustrated in figures 1 and 4, especially in the thymus in which the myeloid transformation is not very active in the normal state.

Figures 14 and 15 illustrate another type of heterophil myelocyte seen especially

well in the imprints. Their nuclei have the structure of the small or medium lymphocyte, the intermediate stages in their development from lymphocytes were seen and have been described. In the sections most of the heterophils, like those of figures 11 to 13, are derived from large basophilic lymphocytes. It is possible, however, that cells 12 and 13 of the sections correspond to cells 14 and 15 of an imprint, although there seems to be too much condensed chromatin in cell 15 for the nucleus of a large lymphocyte.

The heterophils of figure 10 which have originated from the fixed mesenchymatous reticulum have nuclei which suggest that these cells correspond to cells of figures 1 and 2 of the imprints. In the imprints the mesenchymatous tissue has nuclei similar to the one of figure 7, in sections they appear as in the right-hand nucleus of figure 10.

#### REGENERATION OF LYMPHOCYTES AFTER IRRADIATION

From the character of some of the immature lymphocytes in the normal thymus one would suspect that there was some development of lymphocytes from the mesenchymatous tissue. However, all the transitional stages could not be traced in either sections or imprints. Several fields were located similar to the one shown in figure 10 in which granulocytes were developing from the fixed mesenchymatous tissue, but a similar origin for lymphocytes was not seen in the normal animal. This proves that regeneration of lymphocytes in the normal thymus is homoplastic, although it seems likely that a few are derived from the mesenchymatous portion of the stroma.

The picture is very different during the active phase of regeneration following irradiation. In this material many clear cut instances were seen of the development of lymphocytes from the mesenchymatous reticulum. Sections of thymus fixed in Helly's fluid, stained with methyl green and pyronin, and dehydrated with dioxan were especially good for this problem.

The animal used for detailed study was 4 or 5 months old. It was irradiated with a dosage of 500 r u and killed six days later. Thanks for this are due Dr. Harry W. Mixer of our Department of Radiology. Only the region of the thymus was irradiated. The dosage was probably sufficient to eliminate most of the lymphocytes from the organ.

Sections showed that recovery was not complete and that regeneration of lymphocytes was still in active progress. Mitoses were numerous. Although this was a late stage of regeneration the material was very favorable for study of the local origin of some of the lymphocytes.

The imprints from this animal contained many more large lymphocytes with immature nuclei, similar to those of figures 8 and 9, than were observed in the normal animal of this same age or in the thymuses of the newborn animals. Many of the nuclei were of the blast type, a few suggested origin from nuclei of mesenchymatous reticulum. The increased number of lymphoblasts might be accounted for by the numerous mitoses.

In the sections the boundary between cortex and medulla was not very sharp.

and often could not be determined. The cortex of most lobules was very narrow and its outer portion was very dense with closely packed lymphocytes, many of which were of the large variety. Mitoses were especially numerous in this region, and it was here that transitional stages between fixed tissue cells and lymphocytes were most numerous. The intermediate stages were similar to those of figure 10 except that the lymphocytes did not develop granules. Sections of this same material stained with Dominici showed that no new myelocytes were being formed and that those present were degenerating. Comparison with normal material leads to the conclusion that exposure to x-rays reduces or suppresses the local production of granulocytes.

Not all of the fixed tissue of the subcapsular region is of mesenchymatous origin, and it is only in a few limited areas that one can see the development of lymphocytes from the fixed tissue the characteristics of which have already been described. Islands of epithelial reticulum show no signs of activity, and this is also true of the compressed epithelium between the numerous lymphocytes of the peripheral cortex.

The lymphocytes originating from the mesenchyme are mostly large and they soon develop very basophilic cytoplasm. Their nuclei may still have the structure of the mesenchymatous nuclei and have very little chromatin. The cell outline usually is quite irregular but tends to become more rounded as the cytoplasm increases in amount and density.

Small and medium lymphocytes which have been formed in the outer portion of the cortex are crowded towards the medulla in dense strands between the radially arranged blood vessels. In the outer portions of the medulla of some lobules these strands may spread out to form irregularly shaped masses of densely packed lymphocytes. Similar dense groups of small lymphocytes may also occur deep in the medulla and in the cortex. They may also form dense collars about some of the blood vessels. This distribution of lymphocytes was also noted by Christensen and Griffith<sup>62</sup> during accidental involution and early regeneration in rats.

Lymph vessels which usually accompany the larger blood vessels are often packed solidly with small and medium lymphocytes. Rudberg<sup>25</sup> thought this meant that during regeneration many of the lymphocytes immigrated through the lymph vessels, possibly from neighboring mediastinal lymph nodes which regenerate faster than the thymus. Both he and Hart<sup>24</sup> stated that the number of mitoses would not account for the rapid production of lymphocytes. Rudberg, although he worked with many x-rayed animals, could not see any evidence for migration of lymphocytes from the lymph vessels to the surrounding thymus tissue, and this is also true of the material being described in the present study.

The development of lymphocytes from the fixed tissue also occurs in the medulla in regions containing abundant connective tissue, large blood vessels, and often Hassall's corpuscles. Here it is again evident that the epithelial tissue which forms the corpuscles of Hassall is in no way associated with the production of lymphocytes.

The relative number of large lymphocytes is not as great in the medulla as in the

peripheral cortex. The lymphocytes developing from the fixed tissue are mostly of medium size and they originate from smaller mesenchymatous cells, with smaller nuclei than those of the cortex.

The other irradiated rabbit was an old animal in which the thymus was in an advanced stage of involution. The region of the thymus was exposed for a dosage of 400 r u and the animal was killed 8 days later.

The regeneration of lymphocytes is not as extensive as in the younger animal though the time interval between irradiation and death was two days longer.

There is marked hyperplasia of the epithelium which forms wide marginal bands in some places and large islands in others. The latter usually extend to the surface but may be central. These epithelial marginal bands and islands which form during the involution of the thymus were described in detail by Bienert (1923).<sup>53</sup>

Most of the lymphocytes are concentrated in dense masses which usually have a central location but may be more peripheral and extend to the outer margin of the long narrow organ. It is difficult to see any reticulum in these regions. Epithelium of the islands and marginal bands forms a dense nucleated mass without cell outlines.

There are a few scattered lymphocytes and small groups of them in the epithelium. It was impossible to detect any mesenchyme associated with the epithelium which could give rise to lymphocytes. Large blood vessels are surrounded by connective tissue. Development of lymphocytes from this tissue could not be detected. A few lymph vessels are filled with lymphocytes, and this may be the chief source of the new lymphocytes, as there is no evidence for their formation from local fixed tissue in this animal.

Rudberg<sup>25</sup> states that after intense radiation which destroys all the lymphocytes the first ones to reappear enter the central portion of the organ where they proliferate and are later distributed to the cortex. Conditions in this animal seem to support Rudberg's conclusion. It is possible that in the older animals the mesenchymatous tissue, if present, is no longer capable of producing lymphocytes. More extensive material would be required to settle this point. In the young irradiated animal there is good evidence for the active production of lymphocytes in the cortex by mitosis and by heteroplastic development from the mesenchymatous reticulum.

Irradiation does not cause myeloid metaplasia of the thymus as does accidental involution from infections as described by Ssyssojew<sup>26</sup> in the thymus of children. No myelocytes were seen in the thymus of the younger irradiated animal, and only one small group of heterophil myelocytes was located in the older animal. Two myelocytes of hematogenous mast cells with large nuclei were seen, and tissue mast cells with small lymphocytic nuclei were fairly numerous in the older rabbit. A few plasma cells and plasma mast cells were also seen. There were no tissue mast cells in the thymus of the younger animal.

There were many cells which appeared to be macrophages in the sections of the older irradiated animal. They contained flakey, granular material which stained pale blue or green with the toluidin blue of Dominici's stain and light pink or

yellow with hematoxylin and eosin. The nuclei were eccentric, and in general appearance the cells were similar to the macrophages of subcutaneous tissue after colloidal dye injection. The included material resembles the endogenous granular substance derived from mitochondria described and illustrated by Tschassownikow<sup>36</sup> in fixed and free reticular cells of cultures of rabbit thymus. He found the epithelial cells of the cultures to be only slightly phagocytic. The granular cells of my preparations did not contain intact lymphocytes or their nuclei.

Some of the nuclei of the granular cells were of the histiocytic or lymphocytic type, but many had nuclei similar to those of the epithelial cells, including even the spherical nucleoli and nuclear grooves. The nuclear membrane often had many wrinkles. Some of the fixed epithelial cells also contained the same granular substance. The macrophages (?), therefore, seem to be of multiple origin, from lymphocytes, from mesenchymatous reticulum, and from the epithelial reticulum. It is very likely, however, that the technique employed did not permit distinction between true macrophages and epithelial cells. Tschassonikow<sup>36</sup> (1927) could not make the distinction in sections of rabbit thymus, but when he added lithium carmine to his cultures he observed the migration of dye-storing macrophages from the explant. The epithelium of his cultures did not store the dye.

The epithelial origin of macrophages of the thymus was claimed by many authors, among whom may be mentioned Rudberg,<sup>25</sup> Pappenheimer,<sup>13, 14</sup> Hart,<sup>24</sup> Wassén,<sup>54</sup> Ssyssojew,<sup>26</sup> Wituschinski,<sup>27</sup> and Marine.<sup>28</sup>

Popoff (1926,<sup>22</sup> 1928<sup>23</sup>) never saw any macrophages or dye-storing cells developing from the epithelium of cultures and transplants. He derived all the carmine cells from embryonic mesenchyme about the blood vessels. He did not see an intimate mixing of mesenchymatous and epithelial tissues, but believed that the perivascular connective tissue would assume embryonic characters where it came in contact with epithelium. This is contrary to Wassen<sup>54</sup> (1915) who did not see macrophages growing out from the perivascular tissue of cultures of frog thymus, but did see their development from epithelium. The epithelium does not form macrophages or phagocytize degenerating lymphocytes according to Christensen and Griffith<sup>52</sup> who obtained rapid involution in rats fed choline-deficient diets.

Tschassonikow,<sup>36</sup> from his work with cultures, concluded that the reticulum is composed of connective tissue elements and epithelium so intimately blended that they cannot be distinguished except when conditions are unusual, as during involution and in cultures, when the elements may separate and each differentiate in its own particular direction.

Wituschinski<sup>27</sup> (1926) claimed that histiocytes and macrophages which gather about a foreign body introduced in the thymus are derived from adventitial and other connective tissue cells, and from cells of the endodermal reticulum. He also claimed that hemocytoblasts which resemble large lymphocytes are derived from the endodermal reticulum under these conditions. The numerous granulocytes which develop around the foreign body he derived from the hemocytoblasts and adventitial cells of neighboring capillaries. He did not believe that lymphocytes could be derived from the epithelial reticulum.

In the material studied by the writer nothing was seen which would indicate origin of hemocytoblasts or other lymphoid cells from the epithelial reticulum. However, the material does show that the epithelial cells may become free rounded cells, some of which assume the form of unicellular Hassall's corpuscles, while others transform to macrophages, or at least to cells which resemble them very closely.

#### HUMAN THYMUS

Some human material became available during the course of this investigation. It was provided by Dr. Robert W. Collett, who performed the autopsies on infants and children who died in Minneapolis hospitals. The imprints made by Dr. Collett were studied by the writer. Sections were not available at the time this study was made. All of the material shows some postmortem change and was not good enough for detailed study of lymphocyte nuclei. However, it could be seen that the lymphocytes were practically identical to those of the rabbit except that the cytoplasm of the larger cells was not as basophilic. It was noted that the largest lymphocytes did not equal the largest ones of human lymph nodes, and that the thymocytes usually had less cytoplasm than the lymphocytes of lymph nodes.

There were some lymphocytes with leptochromatic nuclei but because of the postmortem changes one could not be certain that they were true 'blast' forms. Cells from the mesenchymatous reticulum, similar to figure 7 from rabbit, could not be identified. Cells from the epithelial reticulum, however, were quite numerous in some cases, especially in a 2 year old male who died of influenza meningitis, in a 10 weeks premature that lived 21 days, and in a 5½ months aborted fetus which lived for 3 hours.

Several groups of 4 to 8 attached epithelial cells were seen, they were most numerous in the premature infant. Most of the epithelial cells, very numerous in the 2 year old child, occurred as single round, irregular or elongated cells. The round cells generally had smooth surfaces, while the margins of the elongated or irregular cells were often serrated or spiked. The nuclei were elliptical in the groups of attached cells and round in most of the free cells.

The nuclear membrane of all the epithelial cells was very thin. Many of these cells had 1 or 2 pale blue spherical nucleoli. In the largest epithelial cells the nuclei were very pale and had little chromatin which was widely dispersed in the form of fine strands and granules. The nuclei of all of the smaller cells had about the same structure, they were darker due to condensation of chromatin in dense short rods and irregular small masses with a considerable amount of pale pink parachromatin between them. The cytoplasm was opaque and nearly homogeneous. It was colored dark gray which often had a pale pink cast with May-Giemsa staining. In some cells it contained some blue flakes which appeared to be similar to the material included in the macrophage-like cells of the older irradiated rabbit. This substance was not abundant in any of the human cells, nuclei were not eccentric, and the cells did not resemble macrophages as they did in the rabbit.

The epithelial cells resist postmortem changes much better than the lymphocytes,



for all showed perfect preservation. Their nuclei and cytoplasm are so characteristic and specific that there was never any difficulty in identifying them. Only a few of these cells had the nuclear grooves that seemed rather characteristic of the rabbit epithelial cells. Many nucleoli were seen in the imprints, but in the rabbit they were seen only in the sections. The nuclei of the human cells are coarser and darker than those of the rabbit. The cytoplasm shows about the same structure and staining reaction in both.

All three types of myelocytes were seen in the human material. They were most numerous in the fetus and least numerous in the 2 year old child that died of meningitis. Most of the eosinophil myelocytes were of the bone marrow type and many had both basophilic and acidophilic granules in the same cell. This difference in staining reaction was also seen in some of the neutrophil myelocytes. These variations in staining were not seen in the rabbit thymus but were noted in the marrow. A few human eosinophils had small, dark lymphocytic nuclei. There were no basophilic granules in these cells. Most of the eosinophils of the rabbit were of this type.

Some macrophages with lymphocytic nuclei were seen in the thymus of the premature infant, and in a stillborn that died  $1\frac{1}{2}$  hours before birth from toxemia. Some material that seems to have been phagocytized was included in epithelial cells of the latter case.

Although the human material was obtained at autopsy done several hours after death much of it was sufficiently well preserved to show great similarity between the human and rabbit thymus. There are some differences in details, as in the minute structure of the epithelial cells, the presence of basophilic granules in many of the human eosinophil and neutrophil thymic myelocytes, and the absence from the human thymus of lymphocytic neutrophil myelocytes similar to the pseudo-eosinophils of figures 14 and 15 from the rabbit.

Whether the human thymus also has immature lymphocytes of the 'blast' type could not be answered from this material. There is no explanation for the absence of mesenchymatous elements like the one illustrated in figure 7. Study of more favorable material will be necessary to determine whether the human reticulum contains less mesenchyme than that of the rabbit, or whether there is some other reason for its absence from the imprints.

#### DISCUSSION AND CONCLUSIONS

It has been shown that in the normal rabbit the thymus reticulum consists largely of epithelial elements. A small amount of mesenchymatous tissue is blended with this epithelial reticulum, but the cells of the two types of reticulum, which differ in embryological origin, retain characteristic morphological differences in the adult rabbit. The two types of reticular cells can be demonstrated in both sections and imprints. This is contrary to the opinion of others who, from the presence of fibers, from embryological studies, or tissue cultures of the thymus (Mietens,<sup>21</sup> Jolly,<sup>21</sup> Hartmann,<sup>7</sup> Tschassonikow<sup>26</sup>) concluded that the thymus stroma must include some mesenchymatous elements, although they could not see them in

histological preparations. The mesenchymatous elements are identical in structure and functional potencies to the reticulum of the other lymphoid organs. This tissue is not confined exclusively to perivascular regions, as Popoff<sup>22-23</sup> seems to think it is, although it is more abundant in these locations, especially in the medulla.

The present study has shown that lymphocytes and granulocytes may develop from the mesenchymatous reticulum, although this type of heteroplastic development is not very active in the normal thymus and is difficult to demonstrate for lymphocytes. During active regeneration following irradiation of the thymus of a young animal many examples of the heteroplastic development of lymphocytes were seen. Some macrophages are also formed from free cells of this tissue.

The epithelium does not form lymphocytes or granulocytes. Some authors who believed that thymocytes (lymphocytes) were derived from the epithelium probably saw transitional stages between the mesenchymatous reticulum and lymphocytes. This is especially true of Prenant,<sup>19</sup> who stated that mitosis in endodermal reticulum results in the formation of lymphoblasts with clear nuclei which divide and form lymphocytes.

Free rounded or irregular-shaped cells are liberated from the epithelium. These were especially numerous in some of the human material where they retained all their specific epithelial characteristics. In the irradiated older rabbit some of them assumed the form of unicellular Hassall's corpuscles, and others appeared to be macrophages which retained some of the nuclear characters of the epithelial cells.

Most students of the thymus problem agree with Rudberg,<sup>25</sup> Ssyssojew,<sup>26</sup> Wituschinski,<sup>27</sup> Marine,<sup>28</sup> and others that the epithelium can form macrophages which phagocytize degenerating lymphocytes during pathologic involution and inflammation of the organ. However, Popoff<sup>22-23</sup> and Tschassownikow,<sup>36</sup> working with transplants and cultures, concluded that dye-storing macrophages are derived from mesenchymatous elements only. It does not seem likely that epithelial macrophages which will phagocytize lymphocytes would not also store lithium carmine. It is generally agreed that the fixed epithelial cells, and the free ones which have not transformed to macrophages, will not store the dye.

The thymocytes are true lymphocytes of various sizes and nuclear structure corresponding to those of the lymph nodes. However, the rabbit thymus contains a few large lymphocytes with very immature nuclei (fig. 8) which resemble those of the rabbit myeloblast and lymphoblast of human lymphatic leukemia. According to Sundberg and Downey<sup>41</sup> these are not present in the rabbit lymph nodes. The thymus contains only a few transitional stages between mesenchymatous reticulum and large lymphocytes. These are numerous in lymph nodes and they are usually large cells, larger than any that occur in the thymus. The absence of the largest lymphocytes from the thymus, therefore, seems due to reduced heteroplastic development of lymphocytes in the normal thymus as compared to lymph nodes. The small, medium and smaller large lymphocytes are identical to the corresponding forms of the nodes. The smallest lymphocytes with very dark nuclei with coarse chromatin blocks and little or no cytoplasm are more numerous in thymus than in node. Scanty cytoplasm is rather characteristic of the thymus lymphocytes.

which seems to support the view of Dustin<sup>10</sup> that the nuclei are of chief importance for the function of the thymocytes

The thymic lymphocytes of different types can develop to granulocytes, and they do this in the normal rabbit and human thymus. They also differentiate to plasma cells and tissue mast cells. There were no basophilic granules in any of the heterophil or eosinophil myelocytes of the rabbit thymus. These dark granules are a conspicuous feature of these cells in the rabbit marrow, and they also occur in the corresponding cells of the imprints of human thymus, where most of the eosinophils are of the marrow type. In the rabbit thymus all the eosinophils seem to develop from small and medium lymphocytes with dark nuclei, and their granules are acidophilic when they first appear. Some of the rabbit granulocytes develop from the local mesenchyme (fig. 10) and this may be the origin of the marrow type of heterophil and basophil (figs. 1 and 4). Heterophils, like those of figures 14 and 15, could not be located in the rabbit marrow or the human thymus. They are fairly numerous in the rabbit thymus.

Study of regeneration of lymphocytes six days after irradiation of the thymus of a young adult rabbit showed that some of the lymphocytes are derived from the local mesenchyme under the capsule and in the medulla. The intermediate stages in the process could be traced. Many lymphocytes seem to enter the organ through the lymph vessels that accompany the larger blood vessels. Radiation seems to interfere with the development of myelocytes in the thymus, as none was found in the younger animal and only a few in the older one. Irradiation was also responsible for the production of many macrophage-like cells in the older animal. Many of these originated from the epithelium, but some had lymphocytic nuclei and others resembled the tissue type of macrophage. The fixed epithelium contained some included material that seemed to have been phagocytized. This gives some support to those who derive true macrophages from the thymic epithelium.

The development of lymphocytes from mesenchymatous tissue could not be detected in the older animal. This may be because regeneration of lymphocytes was not very active in this animal. Some tissue mast cells were seen in the thymus of this animal while in the younger animal the basophils were of the hematogenous type.

Mesenchymatous reticular cells could not be detected in imprints of human thymus. Cells of the epithelial reticulum are fairly numerous. They appear in attached groups or as single round or irregular-shaped cells. The nuclei of all these cells are of similar structure which is characteristic of the epithelial cells. Some of the nuclei have a surface groove and most have spherical nucleoli.

All three types of promyelocytes and myelocytes are present in the imprints of human thymus. They are similar to those of bone marrow with the exception of some of the eosinophils which develop from small lymphocytes.

The mature lymphocytes are similar to those of the rabbit, and, as in the rabbit, the largest lymphocytes of nodes do not seem to occur in the human thymus. The material was not adequate for determining whether the human thymus also has lymphoblasts or reticular lymphocytes.

## SUMMARY

Thymocytes are genuine lymphocytes. The largest lymphocytes, of lymph nodes do not occur in the thymus, the smallest ones with pacychromatic nuclei and scanty cytoplasm are more numerous in the thymus. Some lymphoblasts similar to myeloblasts occur in the rabbit thymus.

All three types of granulocytes develop from lymphocytes and to some extent from the mesenchymatous reticulum in the rabbit. In the rabbit the heterophil and eosinophil granules are acidophilic when first formed, while in the human thymus many have a basophilic quota as in the marrow. The reticulum is largely of epithelial origin and retains its epithelial characteristics in cytoplasm and nucleus. In the rabbit some mesenchymatous tissue is blended with the reticulum and may give origin to lymphocytes and granulocytes. The epithelium may form macrophages but does not differentiate to lymphocytes and other types of cells. In imprints from the rabbit the epithelial cells usually can be distinguished from those of the mesenchymatous reticulum. During regeneration following irradiation the latter tissue can be seen to form some lymphocytes locally, other lymphocytes enter the organ through the lymph vessels.

Imprints stained with May-Grunwald and Giemsa are of great value for detailed cytology of the thymus and for determining cell relationships. Sections of thymus fixed in Helly's fluid, stained with methyl green and pyronin and dehydrated in dioxan are excellent for lymphocytes and the transitional stages resulting from their development from fixed mesenchymatous tissue during regeneration of the organ following exposure to x-rays.

## ADDENDUM

Since this paper was written and sent to the editor an important paper on "Pure cultures of rabbit thymus epithelium," by R. G. Murray, has been published (*Am. J. Anat.* 81: 369, 1947). By cutting away the main part of the explant and leaving only an epithelial outgrowth, Murray was able to obtain a pure culture of the epithelium. It was found that this epithelium grows like that from other sources. At times it grew like connective tissue, although mesenchymatous tissue could be excluded in this case. 'Pinocytosis' and possibly phagocytosis are characteristics not common to other epithelia. True macrophages, however, were not formed from the thymus epithelium. Evidence was not obtained for an epithelial origin of the rather numerous macrophages seen in the early and late cultures.

The thymocytes behaved like true lymphocytes and some of them developed to myelocytes with lymphocytic type of nucleus.

In two instances, mitoses of epithelial cells resulted in the formation of one daughter cell resembling a thymocyte, while the other one retained the epithelial character of the parent cell. This is the only evidence obtained from the cultures in favor of a possible origin of thymocytes from the epithelium.

In another recent paper, R. N. Baillif (*Anat. Rec.* 100: 16, 1948), concludes that in the involuting rat thymus, after injections of colloidal chlorazol black E and

mercuric sulfide, the epithelium differentiates directly to thymocytes and to macrophages which ingest the injected material. Most of the new lymphocytes produced during regeneration arise through transformation of epithelial cells.

It is possible that these conclusions can be explained by failure to distinguish between the epithelial and mesenchymatous portions of the thymus stroma.

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# THE EFFECT OF ENDOCRINOPATHIES ON THE BLOOD

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WITH the rapid increase of knowledge of the many functions and interrelations of the endocrine glands, it is natural that their influences on the formed elements of the blood have received extensive study. The essential problem has been to determine just how much the elements of the blood are under direct endocrine regulation and how much they are affected by general metabolic alterations produced by hormones. Attempts to isolate a specific hemopoietic hormone or hormones have not been successful. Extracts of the anterior pituitary gland have been said to aid in the regulation of erythrocyte production<sup>1</sup> and certain steroids of the adrenal cortex are known to affect lymphoid tissue.<sup>2</sup> In this paper we propose to review some of the clinical and experimental evidence pertaining to this subject and to present some of our observations in an attempt to evaluate the importance of hormones in the regulation of blood production.

## I. GONADS

A sex difference in the number of red blood cells and the concentration of hemoglobin has been well established for human adults. Exact figures vary but all authors agree that the values for the male are significantly higher than for the female. One authority<sup>3</sup> states that the red blood cell count averages 4.8 million in females and 5.4 million in males. The concentration of hemoglobin shows a corresponding difference. This difference cannot be ascribed to blood loss in menstruation because a wide variety of mammals and even birds appear to have a well substantiated sex difference.

A mild anemia occurs after castration in the male hamster,<sup>4</sup> rabbit,<sup>5</sup> rat<sup>6,7</sup> and chicken.<sup>8</sup> The anemia is usually slightly hypochromic and microcytic. The administration of androgens, in general, has proved effective in restoring the red blood cell counts to normal or above normal. McCullagh and Jones<sup>9</sup> have found a slight to moderate reduction in erythrocytes and hemoglobin in eunuchoid men. They showed that treatment with testosterone caused a rise in the red blood cell counts and hemoglobin, with cessation of therapy there was a reversal of these changes. An increase in the basal metabolic rate seemed to parallel the improvement in the blood picture. From these data they concluded that the sex difference in basal metabolic rate and in erythrocytes might be a related phenomenon.

Ovariectomy of rats causes a rise in the red blood cell count and hemoglobin to nearly the levels maintained by castrated males.<sup>6</sup> The administration of estradiol to these animals yields values comparable to those of normal female animals. The chicken seems to respond somewhat differently in that the red blood cell count of

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ovariectomized hens is not significantly different from that in the hen. A depression or erythropoiesis follows large doses of estrogens in several species. A moderate to severe anemia has been produced in dogs with both natural and synthetic estrogens.<sup>10-12</sup> The depression is not limited to the red cell series but a severe and sometimes fatal granulocytopenia or thrombocytopenia will occur on continued treatment. Monkeys seem to be much more tolerant of similar doses and show only a slight anemia.<sup>12, 13</sup> Hematologic complications of estrogen therapy in humans seems to be rare.<sup>\*</sup>

There is not universal agreement as to sex differences in the number of platelets. A slightly lower average platelet count has been reported for women by Pohle.<sup>14</sup> In 13 normal women it was found that a gradual decrease in platelets occurred during the fourteen days prior to menstruation which was followed by a rapid return to normal or increase after the onset of menstruation. Purpura hemorrhagica is found more frequently in females and cases of thrombocytopenic purpura have been described in which the purpuric episodes recurred only at the time of menstruation.<sup>15</sup> These observations suggest that platelets are influenced by certain female sex hormones or perhaps by the menstrual toxin described by Smith and Smith.<sup>16</sup>

## 2. THYROID

Considerable clinical and experimental evidence indicates that the thyroid hormone has a definite influence on hematopoiesis. An anemia occurs with regularity in many laboratory animals following complete thyroidectomy. The changes which occur in the rat<sup>17</sup> and rabbit<sup>18, 19</sup> have received the most thorough study. The characteristic picture is a moderate anemia which is normochromic and slightly macrocytic. Gastric acidity in the rabbit is unchanged and megaloblasts do not occur in the bone marrow. A diminished ability to regenerate red cells and hemoglobin following a standardized hemorrhage has been demonstrated in thyroidectomized rats.<sup>20</sup> The defect was corrected by thyroxine, cobalt and testosterone, suggesting that the disturbance in blood regeneration is nonspecific.

Anemia is frequently observed in patients with myxedema. Emery<sup>21</sup> attributed the first definite description of this feature of hypothyroidism to Charcot in 1881. This was seven years after Gull's original paper describing the disease. The report of the London Clinical Society on myxedema in 1888 added that allied with the fall in body temperature are changes in the blood. There is not only anemia due to loss of corpuscles, but the relative proportions of these constituents are also altered.<sup>22</sup> Following these early observations, many other authors have confirmed and extended knowledge of this anemia. The importance of the recognition of this cause of anemia was emphasized in 1921 by Dr. Minot<sup>23</sup> in a clinic at which he presented two curable cases of anemia. The changes in the blood were summarized and the beneficial effects of treatment were described. In commenting on the etiology of the anemia he said: "The anemia in this case was apparently dependent upon a decreased formation of blood. This decreased activity of the marrow is

\* One patient has been found to have repeated attacks of agranulocytic angina manifested on the first day of the menstrual cycle.<sup>24</sup>



entirely consistent with the diminished activity of the other functions of the body "

Stern and Altschule<sup>24</sup> have described the blood changes in human beings under conditions which approach in simplicity the animal experiments referred to above. Their patients were subjected to total thyroidectomy for the relief of angina pectoris or congestive heart failure. An anemia of some degree was common and the onset of anemia seemed to coincide with the drop in the basal metabolic rate. There was a slight increase in mean cell volume and in color index. Some decrease in white blood cell counts occurred, but the differential counts remained unchanged.

Anemia in spontaneous myxedema has been found by Bomford<sup>25</sup> to be of three types. A slightly macrocytic variety of moderate severity commonly occurs. Similar to the anemia following thyroidectomy, it is characterized by a slight macrocytosis and increase in color index. It differs from pernicious anemia in that there is little poikilocytosis or anisocytosis of the erythrocytes and the bone marrow is hypoactive. Gastric function may or may not be normal. No reticulocyte response follows treatment with liver or iron, but the anemia slowly disappears on prolonged treatment with desiccated thyroid.

Some cases of myxedema may be associated with a hypochromic anemia of varying degree. Splenomegaly, a smooth tongue and changes in the nails are sometimes observed. The blood smear resembles iron-lack anemia with the exception that the cells tend to be larger. Achlorhydria is common but not invariable. A reticulocyte response to iron occurs, but complete recovery depends on both iron and thyroid.

Quite rarely Addisonian macrocytic anemia may be a complication of hypothyroidism. In such patients the signs and symptoms of pernicious anemia and combined system disease may be superimposed on the features of myxedema. The blood resembles pernicious anemia except that the color index may be even higher and the cells larger. Maximum improvement depends upon combined liver and thyroid treatment.

Bomford has concluded that the simple macrocytic type is the result of a decrease in size of the erythron as a physiologic compensation for the diminished need of the tissues for oxygen. The bone marrow undergoes hypoplasia, with shrinkage of its total volume. A marked reticulocytosis following treatment is not to be expected as compared to the anemias due to a maturation arrest. The slow return of the peripheral blood to normal values was explained by the gradual resumption of activity and cellularity in the bone marrow. The other two types of anemia are due to deficiencies of iron and liver extract factor apparently dependent on defective gastrointestinal function.

The simple slightly macrocytic anemia of myxedema occasionally is mistaken for pernicious anemia. However, little variation in size and shape of the red cells occurs in myxedema and the multilobed polymorphonuclear leukocytes and bone marrow changes associated with pernicious anemia are absent. The finding of normal gastric juice occurs in about one half of the cases of myxedema. The basal metabolic rate is of considerable aid in diagnosis because it is usually elevated in pernicious anemia. A yellow color of the skin may be common to both diseases, but

this pigment is bilirubin in pernicious anemia and excess carotene in myxedema. The most important differential point, however, is the presence or absence of an adequate reticulocyte response to liver extract therapy.

The frequency of anemia in spontaneous myxedema has been reported by Lerman and Means<sup>26</sup>. Sixty per cent of 52 patients with myxedema had red blood cell counts of less than four million and 52 per cent had hemoglobin concentrations which were less than 70 per cent. Achlorhydria occurred in 53 per cent and was more frequently associated with anemia than was normal gastric acidity. These authors ascribed considerable etiologic importance to these changes in gastric secretion. The coexistence of myxedema and pernicious anemia seems to be more common than could be accounted for on the basis of probability. Means, Lerman and Castle<sup>27</sup> have described 5 such cases responding to liver extract.

Testosterone has been used in the treatment of anemia in myxedema by Glass.<sup>28</sup> He reported the case of a 71 year old man with the classic features of myxedema. The red blood cell count was 2.8 with a color index of 1.1. Liver and iron had been given in adequate doses without increasing the number of erythrocytes. Four months of therapy with desiccated thyroid failed to correct the anemia. On the addition of testosterone and methyl testosterone to the therapeutic regime, the erythropoietic response was prompt and blood counts returned to normal in the course of a few months. An adequate hematologic response to desiccated thyroid might have eventually occurred in this patient, but the stimulating effect of testosterone on erythropoiesis cited above and the observed low excretion of 17-ketosteroids in myxedema provide a rational basis for such therapy. We have employed the combination of testosterone and desiccated thyroid in myxedema with apparent acceleration of blood regeneration, as illustrated by the following case.

*Normocytic, slightly hypochromic anemia associated with myxedema with restoration of normal blood values after treatment with desiccated thyroid, testosterone propionate and ferrous sulfate*

Case 1. M. V., a housewife, aged 47, of Irish parentage entered the Boston City Hospital in May 1945 complaining of weakness which appeared at the birth of her last child five years previously. The baby had been born at full term and the delivery was uncomplicated. Lactation was normal and menstrual periods returned at normal intervals following the cessation of lactation. She did note, however, increased fatigue and weakness. Somnolence and lethargy became troublesome. For one year she had noticed thinning of her hair and dryness of her skin. Bowel movements had continued to be regular. There had been no shortness of breath, numbness or tingling of the extremities, and no soreness of the tongue.

On physical examination the patient was a pale, somewhat poorly developed middle-aged female in no distress. The skin was cool and dry and the fingernails were brittle and spoon-shaped. The hair on the scalp was thin and dry and the axillary and pubic hair was sparse. The tongue was large but possessed normal papillae. The blood pressure was 101 mm. of Hg systolic and 75 diastolic and the pulse was 94. The heart and lungs were not abnormal. The liver and spleen could not be palpated. No abnormal neurologic signs were noted.

Laboratory examinations showed the following. Repeated urinalysis demonstrated only a small amount of albumin on occasions without other abnormality. The serum cholesterol was found to be 204 mg. per 100 cc. The prothrombin concentration was 50 per cent of normal. Carotenoids were demonstrated in the serum by a presumptive test. The basal metabolic rate was -36 per cent. An insulin tolerance test using 2.5 units of insulin, intravenously, did not reveal sensitivity or hypoglycemic unresponsiveness. A water test<sup>29</sup> and a sodium deprivation test<sup>30</sup> were normal, suggesting normal adrenal function. The sella turcica was normal by x-ray.

Examination of the blood showed a red cell count of 2.98 million, and the hemoglobin concentration

was 48 per cent \* The white blood cell count was 7,200 and the differential count showed the following, polymorphonuclear neutrophils 64 per cent, band forms 6 per cent, eosinophils 1.5 per cent, basophils 0.5 per cent, small lymphocytes 15 per cent, large lymphocytes 3.5 per cent, monocytes 7.5 per cent and myelocytes 2.0 per cent. The platelets appeared normal.

The patient was given a diet of about 2,500 calories supplemented with vitamins in the form of Vegex, 90 cc per day, 2.5 mg of thiamin, 50 mg of ascorbic acid and 50 mg of nicotinic acid a day. Desiccated thyroid was begun soon after entry in a dose of 32 mg and was later increased to 96 mg. Ferrous sulfate was administered in a daily dose of 0.9 Gm for a week. Two cc of purified liver extract (Lilly) containing 30 USP units was given in a single dose by intramuscular injection. Five cc of crude liver extract (Wilson) was administered intramuscularly daily for five days. No significant increase in

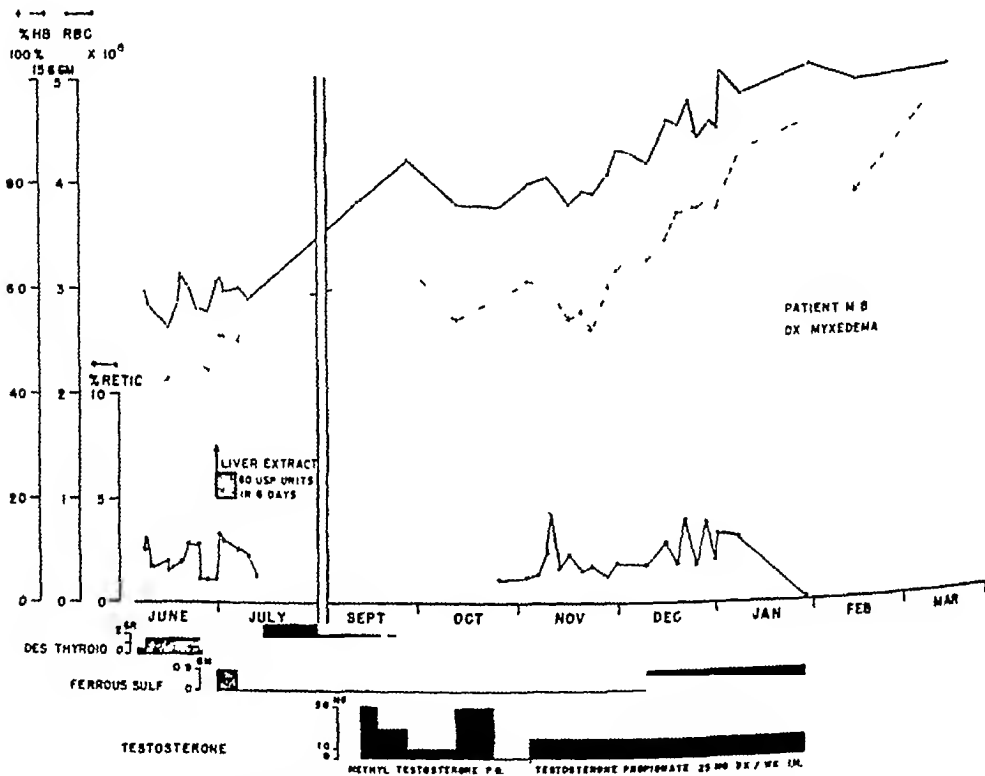


FIG. 1. EFFECT OF THERAPY ON THE RED BLOOD CELL COUNT AND HEMOGLOBIN CONCENTRATION IN CASE 1.

reticulocytes was observed following any of the above therapies. The time relations of the various medications and the hemarologic response are shown in figure 1.

At the time of the patient's discharge from the hospital, the red blood cell count was 2.9 million and the hemoglobin concentration was 56 per cent. She had noted improvement in strength and general well-being. The basal metabolic rate had risen to 18 per cent. She was followed at intervals in the outpatient clinic. From September 13 to October 27, 1945, she received a total of about 1400 mg of methyl testosterone in daily doses of 10-50 mg without improvement in her blood. In November 1945 she was readmitted for more intensive treatment. At this time it was noted that axillary hair was not present and that the spoon-shaped deformity of the nails was absent. The possibility of gastrointestinal bleeding as a cause for her refractory anemia was investigated. Repeated stool examinations were negative for occult blood. The basal metabolic rate was plus 10 per cent. In addition to desiccated thyroid, testosterone\*

\* One hundred per cent hemoglobin is equivalent to 15.6 Gm per 100 cc for all determinations from this laboratory.

propionate was administered three times a week in a dose of 25 mg by intramuscular injection. Ferrous sulfate, 0.75 Gm per day, was given for the last two weeks of her hospital stay. A progressive improvement of red blood cell count from 3.9 to 4.7 million occurred and the hemoglobin concentration increased from 65 per cent to 75 per cent. The same therapy was continued after discharge from the hospital and examination of the blood on January 28, 1944 showed normal values.

*Comment* This woman noted the onset of symptoms of hypometabolism following delivery. However, there was no abnormal bleeding or shock and the history of normal lactation and the reappearance of regular menstrual cycles indicates that if postpartum necrosis of the pituitary was the cause of her hypothyroidism, there was no panhypopituitarism. However, the laboratory data are consistent with primary thyroid myxedema. She presented a moderately severe normocytic, hypochromic anemia which did not respond to therapy with vitamins, liver extract and iron. The hemoglobin had risen slightly after five months of treatment with desiccated thyroid. The addition of testosterone propionate by intramuscular injection seemed to accelerate blood regeneration. The combination of iron, testosterone propionate and thyroid proved effective in restoring the blood to normal.

The changes in the blood in hyperthyroidism have received much study. The early clinicians believed that anemia was an important feature of the disease and in particular emphasized the association of hyperthyroidism with so-called 'chlorosis'. With improvement in methods for counting red blood cells and estimating hemoglobin and the systematic application of these methods to large groups of patients, it became apparent that some of the features of thyrotoxicosis which had suggested anemia had been misinterpreted. In the first comprehensive study of the blood in this disease by Kocher,<sup>31</sup> the red blood cell count was generally normal and sometimes even above the levels then accepted as normal. Subsequent work by many authors has sustained this finding.

Changes which occur in the white cells have given rise to a great deal of study and speculation. After examining the blood of 106 patients with thyrotoxicosis, Kocher<sup>31</sup> described in 1908 what he believed to be the pathognomonic blood picture in this disease. He emphasized the following features: a tendency toward leukopenia with a reduction mainly of polymorphonuclear neutrophils, both a relative and absolute increase in lymphocytes, a moderate increase in eosinophilic leukocytes. The changes in the white blood cells were claimed to be a reliable index of prognosis and the return to normal was believed to be a valuable indication of successful treatment.

Most subsequent investigators have been unable to confirm the significance of many of the features of the Kocher blood picture. Leukopenia was found to be usually slight and frequently absent. In general most of the reports have confirmed the tendency toward lymphocytosis, but the diagnostic usefulness of this change was lost when many other causes of lymphocytosis were recognized.

Recently Bistrom<sup>32</sup> has carefully reinvestigated this problem. He made a comparative study of the morphology of the peripheral blood and bone marrow in patients with nontoxic goiters, toxic goiters and in control subjects admitted to the hospital for minor surgical procedures. The peripheral blood and bone marrow of patients with nontoxic goiters did not differ significantly from the control group.

In the group with thyrotoxicosis there were 33 patients. A moderate reduction in red cells and hemoglobin occurred in 10. The white blood cell counts were low or normal and a relative granulocytopenia and lymphocytosis were found on smear. The lymphocytic infiltration of the thyroid gland which was examined after surgical extirpation was correlated with the degree of lymphocytosis. Only minor changes suggesting greater immaturity of the predominant cells were found in aspirations of sternal marrow.

Treatment with iodine was followed by a return toward a more normal differential white blood cell count, but the three groups responded to an operation with about the same changes in the blood. The author concluded that the changes occurring in thyrotoxicosis were slight and inconstant and could not be of aid in diagnosis or in estimating prognosis. Other recent reports agree with this thesis.<sup>33, 34</sup>

Probably associated with the lymphocytosis which may occur in thyrotoxicosis is the hyperplasia of lymphoid organs which occasionally is present. A generalized enlargement of the lymph nodes and spleen and a persistence of the thymus have been described.

The changes which occur in lymphocytes and lymphoid tissues have received many interpretations. It has been attributed to a constitutional abnormality not directly due to the thyroid.<sup>35</sup> A direct stimulating effect of the thyroid hormone on lymphoid tissue has been suggested.<sup>36</sup> Menkin<sup>37</sup> believed that the relative lymphocytosis in cases of exophthalmic goiter was due to sympathetic stimulation of lymphoid structures, particularly the spleen.

All authors do not agree that the lymphocytes are significantly increased. Hertz and Lerman,<sup>38</sup> using supravital staining technics, concluded that the most marked and characteristic finding in the blood of patients with exophthalmic goiter was a relative and absolute monocytosis. The lymphocytes were found to be normal in absolute numbers. The discrepancy of their observations as compared with the findings of others was attributed to the greater accuracy of the staining technic that they used.

In recent years the influence of adrenal hormones on lymphocytes and lymphoid tissue has been emphasized (*vide infra*). It is now evident that certain adrenal steroids oxygenated on the eleventh carbon atom cause a decrease in the number of circulating lymphocytes and a decrease in size of the thymus and lymph nodes. Selye<sup>39</sup> has described an apparent antagonism between the thyroid hormone and adrenal hormones in controlling the size of the thymus. No significant decrease in the weight of the thymus of rats occurred after thyroidectomy, suggesting that this hormone is not requisite for the persistence of the thymus. However the induction of stress in such rats by means of formaldehyde injections was followed by a severe and often fatal alarm reaction. The involution of lymphatic organs and thymus was much more marked than in intact animals. It appears, therefore, that animals which have been thyroidectomized are greatly sensitized to the action of adrenal hormones.

From these observations it would seem reasonable to suppose that the lymphocytosis and persistence of thymus which has been described in thyrotoxicosis

might be the expression of a relative deficiency of adrenal steroids. Some evidence that such a deficiency exists has been obtained in this laboratory.<sup>40</sup> Despite the severe stress imposed on patients with thyrotoxicosis, the excretion of 'cortin' as measured by a chemical method is frequently markedly subnormal. This observation has been interpreted tentatively as indicative of increased breakdown of hormone, although more studies are necessary to establish this as a fact.

### 3 THE ADRENAL CORTEX

Great advances have been made in the understanding of the physiology and chemistry of the adrenal cortex. The adrenal secretes several substances which have unrelated and even antagonistic actions. One of the most interesting phases of adrenal physiology has been the demonstration of the influence of the adrenal on lymphocytes and the release of antibodies.

In all laboratory animals adrenalectomy results in death after a short period if replacement of salt and/or hormones is not provided. Adrenalectomized rats show an increase in polymorphonuclear leukocytes and lymphocytes about twenty-four hours before death.<sup>42</sup> An increase in the weight of the thymus and systemic lymph nodes has been reported in adrenalectomized rats maintained in good condition by means of the addition of sodium chloride to their drinking water.<sup>42</sup> In cats surviving a relatively long time after operation there is a decrease in polymorphonuclear cells and an increase in lymphocytes.<sup>43</sup> Increases which have been reported in the red blood cell count and hemoglobin concentration are indications of the disturbed electrolyte and fluid metabolism which causes marked hemoconcentration.<sup>44</sup> If adrenalectomized cats and dogs are maintained in fair general condition by several different methods of treatment, the peripheral blood is essentially normal.

Adrenal insufficiency in human beings produces changes similar to those in experimental animals. During an adrenal crisis there is usually a slight increase in red blood cell count and hemoglobin and a relative lymphocytosis. Restoration of normal blood volume by the use of desoxycorticosterone and saline solution frequently reveals a mild underlying anemia. A relative lymphocytosis may persist even after treatment.<sup>46</sup>

Polycythemia has been a frequent, but not a constant, finding in Cushing's syndrome.<sup>47, 48</sup> Approximately one-half of the well authenticated cases have shown an elevation of the red blood cell count.<sup>49</sup> Gunther<sup>50</sup> has collected data on 7 patients with hyperadrenalcorticism with red counts in excess of six million. The abnormality is usually mild and associated mainly with rapid progress of the disease. In itself it does not seem to produce the complications which are observed in polycythemia vera. Many patients with Cushing's syndrome are suspected of having polycythemia in whom subsequent blood counts fail to confirm the clinical impression. A plethoric appearance of the cheeks is common and is due to atrophy and stretching of the skin which permits the transmission of the color of the underlying venous plexuses. Combined with the marked deposition of fat about the face and neck, the red cheeks constitute the pathognomonic facies described by Cushing.

White and Dougherty<sup>2 51</sup> have studied the effects of adrenotrophic hormone and adrenal cortical steroids on red cells and hemoglobin. A single injection of either substance will cause a transitory increase in the red blood cell count, followed by a decrease to lower than pretreatment levels. The continued injection of adrenotrophic hormone resulted in a significant increase in red blood cell count and hemoglobin. The authors concluded that 'it is possible that repeated hormone injection eventually leads to a stimulated production of red cells in an effort to compensate for the diminution of erythrocyte count produced by a single dose of hormone'.

In a number of experimental conditions the size of the thymus varies inversely with the activity of the adrenal cortex. This has been most clearly demonstrated in the response of an animal to stress. Selye<sup>39</sup> has introduced the concept of the 'alarm' reaction as a phase in the general adaptation of the body to harmful stimuli. An increase in the size and activity of the adrenal cortex is a fundamental element in this process. One of the most striking and constant changes is the involution of the lymphatic organs. The loss of weight is most marked and rapid in the thymus. Here the characteristic cells of the parenchyma, the thymocytes, actually disintegrate and twenty-four hours after the onset of a severe alarm reaction only the debris of their chromatin is left lying partly free in the reticulum, partly in phagocytes which are engaged in removing it. The lymph nodes also show signs of involution but without any noticeable hyperplasia of the reticulum. The involution in them usually begins in the germ centers which may disappear almost completely. That the involution of the thymus is secondary to the adrenal cortical activity seems probable because no change occurs in adrenalectomized animals. The injection of certain adrenal steroids into intact animals or adrenalectomized animals, and the injection of adrenocorticotrophic hormone into intact or hypophysectomized, but not adrenalectomized, animals, will reproduce similar changes in lymphoid tissue and thymus.<sup>52 53</sup>

Dougherty and White<sup>2 54</sup> have confirmed and extended these observations by reporting a remarkable decrease in the circulating lymphocytes after the injection of adrenocorticotrophic hormone or cortical steroids into the mouse, rat, rabbit and dog. This phenomenon has been termed 'lympholysis'. Lympholysis in the mouse is particularly striking. Within an hour after the injection of adrenocorticotrophic hormone there occurs a definite fall in the circulating lymphocytes which reaches a maximum in six to nine hours. Recovery to normal takes place within twenty-four hours. Probably considerable variation in sensitivity of lymphocytes to lysis exists because in our laboratory<sup>55</sup> rats of the Sprague-Dawley strain have shown little lymphopenia after injection of aqueous adrenal cortical extract.

Minor changes in chemical structure have been shown to modify the action of various adrenal steroids on lymphocytes. 11-Desoxycorticosterone has been demonstrated to be without effect.<sup>2</sup> On the other hand Compound E (11-dehydro-17-hydroxy corticosterone) and other active steroids with an oxygen on the eleventh carbon atom possess this property.

The destruction of lymphoid tissue is believed to be the source of the increase in

serum beta and gamma globulins which follows injections of active hormones of the adrenal cortex. Direct analysis of lysed lymphoid tissue washed free of all blood serum has demonstrated proteins which are indistinguishable from serum beta and gamma globulins.<sup>56</sup> In immunized animals the lymphocyte contains antibodies which are liberated into the blood stream by the process of lympholysis.<sup>57</sup> Dougherty and White<sup>54</sup> believed that the above mechanism is the explanation for the anamnestic reaction which occurs in acute infection.

The effect of adrenal steroids on the lymphocytes of human beings has not been reported in any detail. Dougherty and White<sup>2</sup> reported observations on several patients with lymphocytic leukemia and also in control subjects. A suggestive drop in the lymphocytes was observed in some of the patients. Forsham et al.<sup>58</sup> observed the effects induced by synthetic 11-dehydrocorticosterone acetate on the lymphocytes. In thirteen experiments on patients with Addison's disease 20-60 mg. of the compound were given as a daily dose. No significant decrease in the lymphocytes was found. It was noted, however, that there was an increase in urinary uric acid excretion in comparison to creatinine. These data seem to suggest that a tissue rich in nucleoproteins was being broken down and it is therefore possible that there was significant 'lympholysis' which was not manifest in the circulating blood. Perera et al.<sup>59</sup> have used the same compound and found an inconstant decrease in the lymphocytes following administration to human beings. Recently Thorn's collaborators<sup>60</sup> have presented interesting observations on the effect of adrenocorticotrophic hormone on the blood picture of man. In normal subjects four hours after the administration of 25 mg. of adrenocorticotrophic hormone by intramuscular injection there was a 90 per cent increase in the absolute number of polymorphonuclear neutrophils, a 40 per cent decrease in lymphocytes and a 78 per cent decrease in eosinophils. Similar changes did not occur in patients with Addison's disease. However the administration of 20 mg. of 17-hydroxycorticosterone to such patients caused an increase of 129 per cent in the absolute number of polymorphonuclear neutrophils, a decrease of 53 per cent in the lymphocytes and a decrease of 76 per cent in the eosinophils.

In our experience little change in the relative or absolute number of lymphocytes follows the administration of adrenal cortical extract in doses of from 10 to 50 cc. to patients with adrenal insufficiency. Figure 2 shows the results which we have obtained on treating a patient with hypopituitarism for nine days. Both adrenal cortical extract (Upjohn) and lipoadrenal cortical extract (Upjohn) were given in doses which are in excess of those required to maintain patients with Addison's disease.

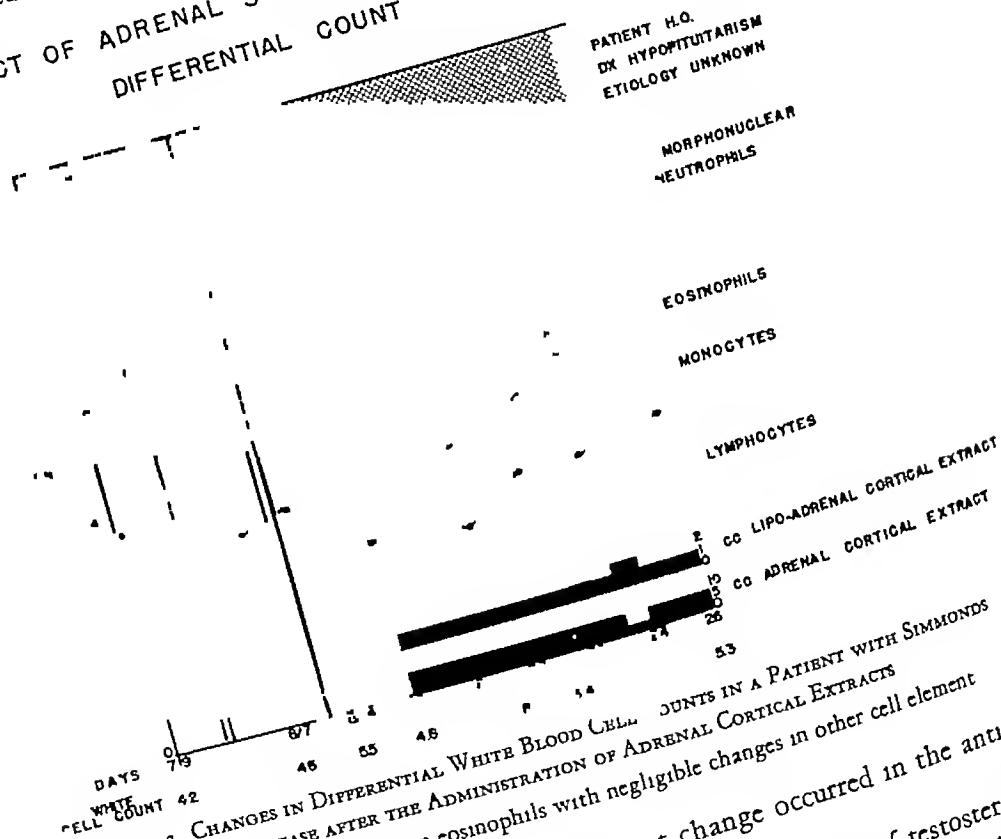
Because of the apparent failure of moderate doses of adrenal cortical extract to alter the blood picture, larger doses were administered. A patient with hypopituitarism secondary to a chromophobe adenoma was selected. Immunization against heat-killed typhoid bacilli was achieved by three injections of 0.1 cc. of a standard vaccine.\* It is interesting that despite the small size of the immunizing

\* This material was very kindly supplied by the Department of Public Health, Antitoxin and Vaccine Laboratory, of the Commonwealth of Massachusetts.



dose the patient noted marked local and systemic symptoms following each injection and that there was a satisfactory rise in antibody titer. Forty cc of adrenal cortical extract was given in two equal doses by intramuscular injection. Figure 3 shows the changes which occurred in the lymphocyte count, the anti-typhoid agglutination titer and the titer of anti-B isoagglutinin (the patient was blood group A). A brisk fall in the number of lymphocytes was noted after six hours with a return to normal after twenty-four hours. A rise in the anti-typhoid agglutination titer was noted at six hours, there was a decline by twenty-four hours and a return

### EFFECT OF ADRENAL STEROIDS ON RELATIVE DIFFERENTIAL COUNT



Note the apparent drop in eosinophils with negligible changes in other cell element to the former titer by six days. No significant change occurred in the anti-B isoagglutinin titer.

The experiment was repeated after the injection of 50 mg of testosterone propionate daily for three days and 25 mg for four days. Following this treatment the anti-typhoid titer seemed to decline. The changes which followed injection of adrenal cortical extract were similar to those observed in this patient prior to testosterone therapy.

We have concluded from these limited observations that the lowering of the lymphocyte counts with a rise in immune bodies, which has been observed in laboratory animals, can be reproduced in human beings, but that relatively large doses of adrenal cortical extract are required.

Other mechanisms of regulation of lymphocytes than pituitary-adrenal control must exist. This is evident from the study of adrenalectomized rats maintained on salt. Crafts<sup>17</sup> found that such animals maintained a normal white count and differential count. De La Balze, Reifstein and Albright<sup>16</sup> reviewed their extensive experience with adrenal disorders in man and could not establish a significant increase in the absolute number of lymphocytes in patients with Addison's disease, although the relative number of lymphocytes was increased.

### EFFECT OF ADRENAL CORTICAL EXTRACT ON LYMPHOCYTES AND ANTIBODIES

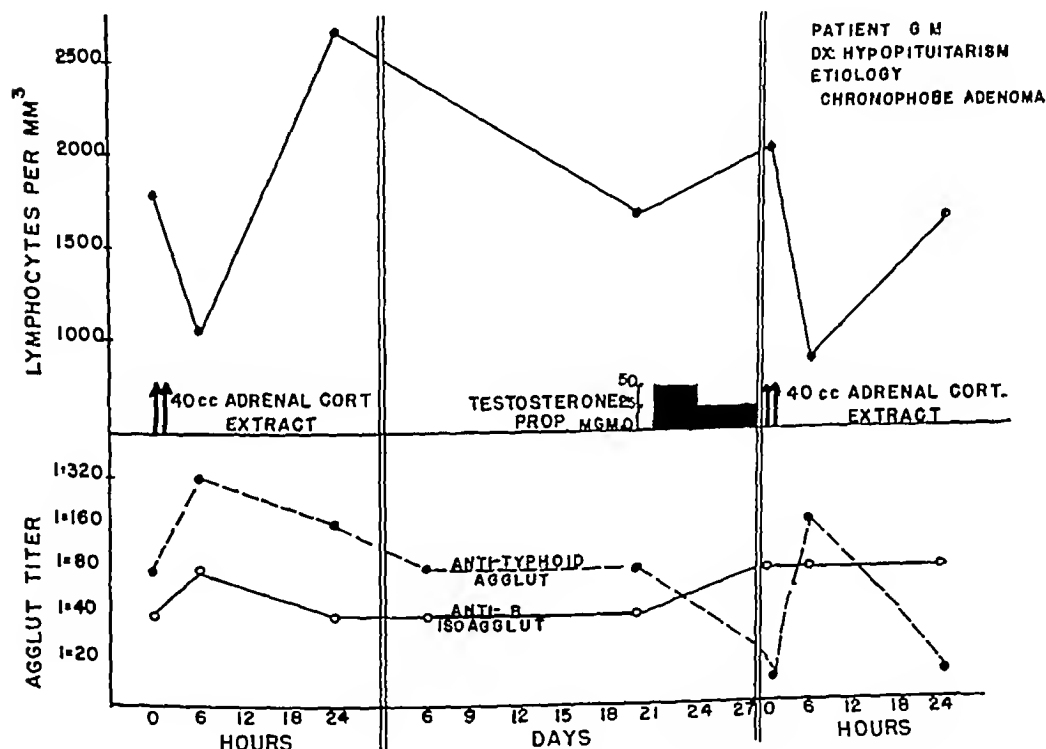


FIG. 3. NOTE THE LYMPHOPENIA AND INCREASE IN THE TITER OF ANTITYPHOID AGGLUTINATION FOLLOWING THE ADMINISTRATION OF ADRENAL CORTICAL EXTRACT.

### 4 THE PITUITARY GLAND

Discussion of the possible mechanisms by which this gland alters hematopoiesis has been reserved until last. It is well known that the anterior lobe of the pituitary gland controls the activity of the other endocrine glands and it could thereby aid in the regulation of formed elements in the blood. It is, therefore, evident that the anemia which is often observed following destruction of the anterior pituitary is a complicated phenomenon.

An anemia following hypophysectomy was first noted by Aschner<sup>61</sup> in dogs in 1912. Later it was determined that rabbits<sup>62</sup> and rats<sup>63</sup> respond to the operation in

a similar manner. Destruction of the pituitary in man frequently results in a moderate anemia.

The decrease in the red blood cell count and hemoglobin which occurs in the hypophysectomized rat, has received the most intensive study. In 1935 Stewart, Greep and Meyer<sup>64</sup> noted that hypophysectomized rats were anemic and that there was a decrease in the number of reticulocytes. A rise in reticulocytes occurred in normal rats exposed to reduced oxygen tension, but not in hypophysectomized ones. Increases in the red blood cell count and hemoglobin of the hypophysectomized rats occurred only if the stimulus of low oxygen tension was applied soon after operation. The reticulocyte response following substitution therapy parenterally has proved an unreliable criterion because reticulocytosis has been produced by many hormonal and nonhormonal agents.<sup>65</sup> In general, reticulocytosis has been unaccompanied by improvement in red blood cell count and hemoglobin concentration. The conclusion was reached that the alterations in the blood following hypophysectomy were due to general disturbances in metabolism rather than the absence of a specific hematopoietic hormone.

On the other hand, the existence of a specific pituitary hormone which stimulates blood formation has been postulated by Moehlig and Bates.<sup>66</sup> The polycythemia of Cushing's disease was attributed to an excessive production of this factor.

On the basis of the response of hypophysectomized rats to oral administration of preparations of anterior pituitary, Flaks, Himmel and Zlotnik<sup>67</sup> postulated the existence of an erythrogenic hormone. They claimed that the anemia of hypophysectomized rats was repaired and that polycythemia was produced by their hormone preparation in intact animals. It is difficult to accept this claim because of the impotency of known pituitary hormones when administered orally.

Beneficial effects on the anemia of hypophysectomized rats have followed treatment with several hormones, pituitary as well as non-pituitary. Vollmer and Gordon<sup>68</sup> and Vollmer, Gordon and Charipper<sup>69</sup> found that testosterone propionate increased the red blood cell count of hypophysectomized male and female rats. Estradiol, on the other hand, seemed to intensify the anemia. Pregnant mare's serum raised the red blood cell count of hypophysectomized male rats, but lowered the counts in hypophysectomized female rats. Thyroxine plus testosterone was moderately effective. Prolactin seemed to be inactive. Desoxycorticosterone produced no definite effect on the bone marrow.

Various types of replacement therapy have been investigated by Crafts.<sup>70, 71</sup> In hypophysectomized female rats the anemia was found to be microcytic and hypochromic and accompanied by hypoplastic changes in the bone marrow. Iron or iron plus copper delayed slightly the onset of anemia. Injection of 0.01 mg. of thyroxine daily maintained a normal erythrocyte count, but did not prevent a decrease in hemoglobin concentration. Histologic examination revealed greater cellularity of the bone marrow and decreased infiltration by fat cells. This was interpreted as an indication of increased activity. The combination of thyroxine, iron and copper maintained a normal red cell count and seemed to increase the amount of hemoglobin. Hypophysectomized adult male rats developed a severe microcytic, hypochromic anemia with hypoplastic changes in the bone marrow. Testosterone

therapy prevented the decrease in the red blood cell count and restored the bone marrow to normal cellularity. Microcytosis and hypochromia were only partially corrected. Because the anemia following castration was much less marked than that following hypophysectomy, the author believed that the anemia in the latter condition was not a manifestation of decreased androgens, although androgens proved partially effective in preventing the experimentally induced anemia.

An anemia following destruction of the anterior lobe of the pituitary in man has been long recognized as a significant feature of the clinical syndrome called Simmonds' disease. Silver<sup>72</sup> reviewed the literature in 1933 and observed that anemia is a constant finding. The hemoglobin averages 50 per cent, with a color index which is usually less than one. Leukopenia is common and an eosinophilia reaching 22 per cent may occur.

Snapper, Groen, Hunter and Witts<sup>73</sup> described 6 cases which they believed presented evidences of pituitary or gonadal deficiency plus an anemia. Case 2 of their series was a woman with pituitary necrosis following hemorrhage and shock at the time of delivery. A macrocytic anemia of 3.2 million red blood cells was present. Case 3 was a patient with hypopituitarism secondary to a chromophobe adenoma. There was a moderately severe macrocytic anemia, accompanied by gastric achylia and evidence of combined system disease. In the other cases hypogonadism was probably primary. The importance of achlorhydria and defective absorption was stressed as the immediate cause of anemia leading to deficiency of either iron or the hemopoietic principle of liver extract. Witts<sup>74</sup> later described two additional cases which he considered to be examples of hypopituitarism and which were associated with a macrocytic anemia responding to liver. He concluded: "The association of pernicious anemia with hyperthyroidism, with pregnancy and with pituitary disease, suggests that there is a hormonal element or mechanism which can lead to the degeneration of the cells which secrete intrinsic factor. We may consider the association of pernicious anemia with hypopituitarism as another example of the precocious senile changes to which the patient with pituitary disease is liable."

The effect of testosterone propionate on the anemia of hypopituitarism has been the subject of a recent report.<sup>75</sup> Two cases of hypopituitarism with anemia are described that responded to the combined administration of testosterone and liver. In one patient at least it is evident that no response occurred with liver extract alone. It was suggested that testosterone enabled the bone marrow to utilize the hematinic principle, which it previously had been unable to do.

Escamilla et al.<sup>76</sup> have reviewed 101 cases of Simmonds' disease, verified by post-mortem examination. Among this group the hemoglobin ranged from 102 per cent to 40 per cent, with an average of 65 per cent. Red blood cell counts ranged from 5.6 to 2.0, averaging 3.7 million. Eosinophilia was commonly observed, with 63 per cent as the average figure.

Sheehan<sup>77, 78</sup> has emphasized the importance of serious hemorrhage and shock at the time of delivery as an etiologic factor producing necrosis of the anterior lobe of the pituitary. The lesion is a thrombotic infarction of variable extent, which later may reduce the anterior lobe to a nubbin of fibrous tissue. This group of

particularly valuable for an understanding of the anemia in Simmonds' disease because the time of onset can be fixed with some certainty and it occurs in young adult females who may live with hypopituitarism for many years. Quite frequently the blood deficit of the precipitating hemorrhage is incompletely restored. However, for the first five years it is not uncommon for these patients to maintain relatively normal red blood cell counts, but with a rather low hemoglobin concentration. During the second five year period there is a definite tendency for the red blood cell count to decrease to between 3 and 4 million, with some increase in the color index. The blood frequently remains at this level indefinitely. Occasionally there is a further decrease in the red blood cell count to a level of from 2 to 3 million with a color index of 0.95 to 1.25. These cases showing a severe anemia and a tendency toward macrocytosis seem to occur in patients with more profound evidences of deficient thyroid function and may be indistinguishable clinically from thyroid myxedema. Leukocytes usually number between 4 to 6 thousand, although leukopenia is common. The smear characteristically shows a relative lymphocytosis with a moderate eosinophilia in about two thirds of the cases.

The results of therapy on the blood in hypopituitarism have received scant attention in the literature. The use of anterior pituitary hormones is the rational approach to the problem, but has not proved successful. Extracts of the pituitary of high potency and purity have not been available. Because of their protein nature, pituitary extracts rapidly lose their effectiveness because of the development of antihormones. Also allergic manifestations, such as urticaria and local pain are common. Because of these disadvantages it has been more practical to use the hormones of the atrophic "end-organ" glands.<sup>79, 80</sup> The combination of desiccated thyroid, desoxycorticosterone acetate and testosterone has provided a reasonably satisfactory method of treatment.

In this clinic the above therapy has been used with various modifications in the treatment of Simmonds' disease. Marked relief from many of the disabling symptoms of this disease has been obtained, but improvement in the blood findings has been inconstant. The following case (case 2) is reported in detail because this patient had been under observation for a period of nine years and during this period adequate trial with many therapeutic agents failed to correct an anemia. Case 3 is reported to show that improvement in the blood may follow hormone treatment without the addition of liver or iron. Hematologic data on 22 patients with hypopituitarism observed during the past six years are presented in table 1.

*Pituitary fibrosis of unknown etiology associated with a moderately severe normocytic and normochromic anemia which failed to respond to liver, iron and hormone therapy*

*Case 2* F. S. (Details of this case have been reported elsewhere.<sup>79, 80</sup>) A white housewife of American parentage, aged 38, entered the Boston City Hospital October 18, 1934 complaining of weakness and vomiting. Following an attack of influenza two years prior to entry, she had been told that she had anemia and low blood pressure. Recovery from this illness was protracted and incomplete and she continued to suffer from asthenia, fatigability, drowsiness and anorexia. A local physician prescribed ground raw liver which she took for a period of about six months without improvement in any of her complaints. She sought hospital care after two weeks of nausea and vomiting. Her past history revealed that she had given birth to a normal child about ten years previous to entry. A hysterectomy was performed some time after delivery for pelvic peritonitis. Unfortunately details of the delivery and operation are not available.

On physical examination she appeared to be an underdeveloped and poorly nourished woman. The skin was pale, smooth and had a yellowish, waxy texture. The tongue had normal papillae. The blood pressure was 92 mm of Hg systolic and 68 diastolic. A mass interpreted as the liver edge was felt on

TABLE I — *Hematologic Observations in Hypopituitarism*

All cases presented clinical and laboratory evidence indicating deficiency of two or more of the following hormones: gonadotropic, thyrotropic, adrenotropic and growth hormone.

## PART I POSTPARTUM NECROSIS OF THE PITUITARY

Case	Duration of Disease, Years	Age	Date	% Hgb 100% = 15 Gm	Erythrocytes $\times 10^6/\text{mm}^3$	% Reticulocytes	M.C.V., cu m crons	M.C.H. micro- micrograms	M.C.H., Conc %	Leukocytes $\times 10^3/\text{mm}^3$	Total Granulo- cytes %	Eosinophils, %	Lymphocytes %	Monocytes %
T R	3	21	4/29/46	68	3.6	0.8	90	29	33	8.6	69	3	21	10
			Given desiccated thyroid 96 mg/day. One pellet (128 mg) DOCA im planted. Testosterone propionate 25 mg intramuscularly daily for 17 days.											
			6/18/46	70	4.1	3.8	87	27	31	4.8				
			Desiccated thyroid continued 10 mg of methyl testosterone daily for one month. Stilbestrol 0.4 mg daily for the next month. Later given 3 courses of Dienestrol for 21 days followed by Progesterol for five days.											
			4/15/47	83	4.6		88	28	32	8.0				
C R	4	23	2/28/47	87	4.5	0.8	93	31	33	6.1	54	0	33	13
A N	12	39	11/9/42	61*	2.8*					8.9*	75*	1*	25*	0*
H O N	14	42	5/28/41	71	3.5	0.2	87	32	37	2.1	72.5	2	20	7.5
			1/17/42	55	2.5	2.0	111	34	31	3.0	62.5	0.5	29.5	8
M T	20	61	9/5/45	55	3.3	1.0	89	25	29	3.1	37.5	3	37.5	34.5
			9/6/45	54	3.1	1.0	92	27	30	3.4				
			Desiccated thyroid 64 mg/day. Three pellets (each 75 mg) of testo- sterone implanted.											
			9/25/45	52	3.1	1.0	94	26	27	3.6				

CLINICAL ABSTRACT T R, F, Severe postpartum hemorrhage, shock (1943). Failure of lactation, fatigue and symptoms of hypometabolism. FSH absent. 17-KS 4.4. C R, F, Severe postpartum hemorrhage and shock (1943). Failure of lactation. Amenorrhea for 2 years then very infrequent menses. Symptoms of hypometabolism. BMR -31. Water test positive. A N, F, Severe postpartum hemorrhage and shock (1930). Pituitary fibrosis found on post-mortem examination. H O N, F (see case 3 in text). M T, F, Onset followed delivery of twins (1925). Fibrosis of pituitary found on post-mortem examination.

\* Determinations carried out by ward laboratories.

deep inspiration at the right costal margin. The spleen could not be felt and there was no lymphadenopathy. The only abnormal neurologic finding was a questionably positive Babinski sign bilaterally.

Laboratory examinations showed the following. The concentration of hemoglobin was 63 per cent and there were 3.35 million red blood cells. The white blood cell count was 4,800 with 66 per cent polymorphonuclear neutrophils and 33 per cent lymphocytes. The hematocrit was 31 per cent. Urinalysis was not abnormal. The Kahn test was at first reported as doubtful but subsequent tests were nega-

There was no occult blood present in the stools. Free acid was not present in the gastric juice even after the administration of histamine. The icteric index was 6 units. The serum cholesterol was 137 mg per

TABLE 1, PART II PITUITARY FIBROSIS OF UNKNOWN CAUSE

Case	Duration of Disease, Years	Age	Date	Hgb 100% = 15 Gm	Erythrocytes × 10 <sup>6</sup> /mm <sup>3</sup>	% Reticulocytes	MCV, cu microns	MCH, micrograms	MCH Conc %	Leukocytes × 10 <sup>6</sup> /mm <sup>3</sup>	Total Granulocytes, %	Eosinophils %	Lymphocytes %	Monocytes %
F S	2	38	1/22/35	70	3.9	0.4	82	28	34	7.8	73	1	24	3
			9/23/36	77	3.6		89	34	37		60.5	3.5	30.5	9
			1/2/41	68†	3.6†		86†	30†	35†	5.4†				
H O	25	53	7/19/46	66	2.9	0.8	111	33	33	4.2	55	9	30	15
			DOCA 2 mg intramuscularly daily. Testosterone propionate 25 mg intramuscularly daily for 11 days, thereafter every other day. Reticulocytes increased to a maximum of 5.6% on 7/28/46.											
			8/14/46	68	3.1	2.0	98	34	35	5.5	57	4	35	8
			Three 75 mg pellets of testosterone and one 75 mg pellet of DOCA implanted subcutaneously.											
			3/6/47	78	3.7		95	33	35	5.2				
A B	29	39	7/21/45	70*	3.0*					7.0*	51*	1*	49*	0*
			Desiccated thyroid, 32 mg daily. Implantation of three 75 mg pellets of testosterone and one 75 mg pellet of DOCA.											
			5/15/47	69	3.5		92	31	33	8.1	60	4	33	7
E J	?	68	7/31/46	68*	3.3*					5.6*	90*	1*	5*	5*
S K	?	69	1/25/46	77*	3.0*					6.1*	69*	0*	29*	2*
			Desiccated thyroid by mouth and DOCA and testosterone propionate by intramuscular injection.											
			2/ /47	82*	4.0*									
A A	?	61	9/28/46	61	3.4		93	27.3	30					
			Desiccated thyroid given in small increasing doses.											
			10/21/46	50	2.5		101	32	31	4.9	44	11	61	3
			Desiccated thyroid, testosterone propionate, liver extract (Lilly) 2500 cc of whole blood.											
				79	4.5	0	91	27	30					

CLINICAL ABSTRACT F S, F, Pituitary fibrosis found at post-mortem examination (see case in text). H O, F, Intracellular calcification. FSH absent. 17-KS 10 mg/day. Insulin sensitive. A B, F, Insulin sensitive. FSH negative. Decreased cortin excretion. E J, F, Pituitary fibrosis at post-mortem. S K, M, Marked insulin sensitivity. FSH absent. Decreased cortin excretion. A A, F, Marked insulin sensitivity. FSH absent. 17-KS 33 mg/day.

\* Determinations carried out by ward laboratories.

† Determinations carried out by Hematology Laboratories, Mass Memorial Hospital. Dr Chester Keefer has kindly consented to the inclusion of these data.

100 cc. A glucose tolerance test was within normal limits. The basal metabolic rate was -26 per cent. X-ray examination of the chest, upper and lower gastrointestinal tract and sella turcica were within normal limits.

TABLE I, PART III HYPOPITUITARISM SECONDARY TO NEOPLASMS

Case	Duration of Disease, Years	Age	Date	% Hgb 100% = 15.6 Gm	Erythrocytes X 10 <sup>6</sup> /mm <sup>3</sup>	% Reticulocytes	M CV, cu m crons	M CH, micro micrograms	M CH Conc %	Leukocytes X 10 <sup>3</sup> /mm <sup>3</sup>	Total Granulo- cytes, %	Eosinophils, %	Lymphocytes %	Monocytes, %
S B	0.5	58	6/22/38 3/29/45	75* 61	3.5* 3.8	0.8	88	25	29	4.2* 5.5	65* 53.5	0* 1.5	32* 34.5	3* 12
M R	1	34		75*						5.1*				
G M	2	42	2/21/44	59	3.6		88	26	29	6.5	68.5	3	19.5	12
Received 5 cc of liver extract (Reticulogen, Lilly) in three injections which was followed by no significant increase in reticulocytes. Testosterone propionate, 25 mg intramuscularly, injected three times a week.														
D N	5	38	4/18/44 3/7/42	88 69	4.4 3.2	0.5	89 99	31 25	35 30					
No reticulocytosis following three daily injections of 10 USP units of liver extract (Reticulogen, Lilly). One pellet (150 mg) of testosterone and six (125 mg) pellets of DOCA implanted. Detailed metabolic studies are described elsewhere. <sup>78</sup>														
P S	6	46	3/31/42 3/29/43	85 95	4.1 4.8		89 91	37 31	32	6.8				
Had received replacement therapy for about two years. <sup>78</sup>														
J C	16	59	8/1/46	70*	4.2*					6.9*	60*		40*	
H A	?	66	7/5/44	67*	4.0*					6.2*	72*		24*	4*
C B	1	43	1/29/46		5.4*					10.5*				
J D	25	20	5/3/44	81*						7.5*				
J B	29	37	8/12/43	76*	4.9*					9.4*				
M H	26	13	1/20/42	80*	4.2*					12.9*				

CLINICAL ABSTRACT S B, M, Pituitary tumor. M R, M, Pituitary tumor. G M, M, Chromophobe adenoma. † D N, F, Pituitary tumor. P S, M, Chromophobe adenoma. J C, M, Chromophobe adenoma. † H A, M, Chromophobe adenoma complicated by annular carcinoma of transverse colon found on post-mortem examination. C B, M, Pituitary tumor. J D, M, Suprasellar cyst with mild hypopituitarism. † J B, M, Suprasellar cyst with moderately severe hypopituitarism. M H, F, Apolar neuroblastoma involving pituitary stalk with hypogonadism and dwarfism. BMR normal. FSH absent.

\* Determinations carried out by ward laboratories.

† Diagnosis established at operation.

A high protein diet with vitamin B complex was given. In addition she received iron, liver and desiccated thyroid separately and in combination for intervals of several weeks without a reticulocyte response.



or improvement in her anemia (see figure 4) Removal of some badly infected teeth seemed to improve her general condition The patient was discharged from the hospital in April 1935

She was seen by Dr Juda Groen in September 1936 at the Boston City Hospital at which time she reported slight improvement in appetite and strength, although she had failed to take desiccated thyroid regularly Signs of myxedema were evident and the absence of axillary and pubic hair was recorded The blood findings were much the same as before

At this time the patient failed to return for further treatment and continued to live a restricted life at home Fatigue and somnolence became increasingly incapacitating until by 1941 she was almost bed ridden Anorexia became almost total and in November 1941 she had an episode of confusion and dis

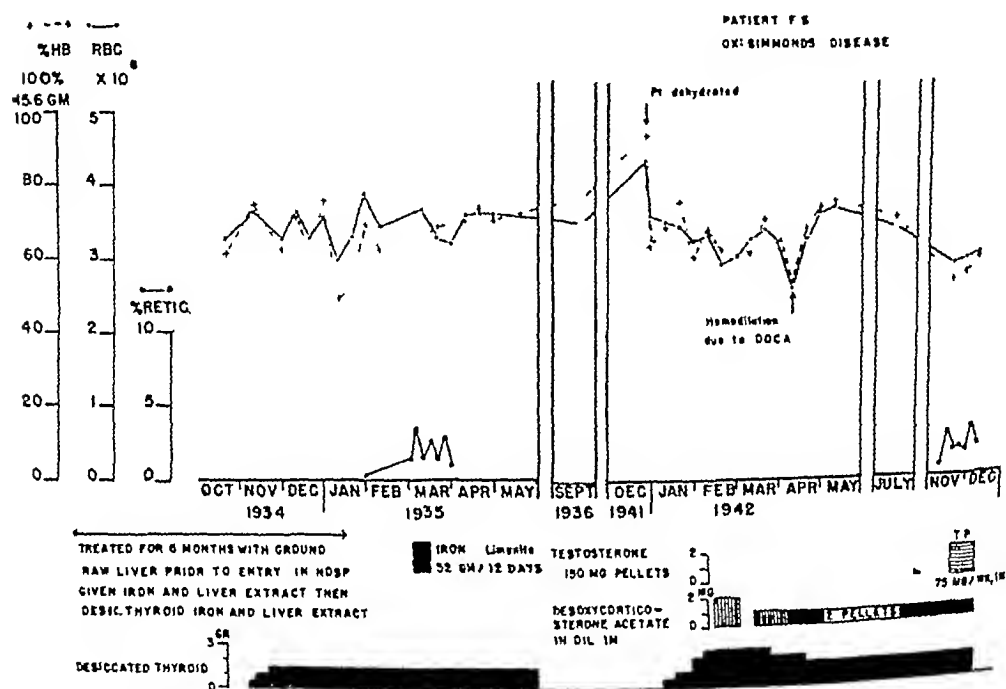


FIG 4 CHANGES IN RED BLOOD CELL COUNT AND HEMOGLOBIN CONCENTRATION IN CASE 2

Data have been plotted as averages for ten day periods Note the apparent absence of response to many different forms of treatment

orientation and because of this she was sent to the Massachusetts Memorial Hospital She was found to be acutely dehydrated and in poor condition on admission Numerous petechiae were present over the right arm The blood pressure was recorded as 108 mm of Hg systolic and 78 diastolic After adequate repair of her dehydration it was evident that her anemia was practically identical to that observed seven years previously The red blood cell count was 3.78 million and the hemoglobin concentration was 65 per cent The white blood cell count on admission was 6,400, with 43 per cent lymphocytes present The platelets numbered 172,000 Aspiration of bone marrow was performed and the following cell distribution was found

Polymorphonuclear leukocytes  
Band forms  
Myelocytes  
Myeloblasts  
Nucleated red blood cells  
Erythroblasts  
Stem cells  
Lymphocytes  
Monocytes

3 per cent  
12 per cent  
30 per cent  
6 per cent  
26 per cent  
8 per cent  
3 per cent  
9 per cent  
3 per cent

No follicle stimulating hormone was found in the urine upon testing for 10 rat units per twenty-four hours. The patient excreted 0.2 mg. of 17-ketosteroids per twenty-four hours. An insulin tolerance test, using 0.05 units of insulin per kilogram of body weight, intravenously, demonstrated moderate hypoglycemic unresponsiveness. A water test<sup>29</sup> was positive, but a salt deprivation test<sup>30</sup> was negative. The basal metabolic rate was -23 per cent.

On January 9, treatment was begun with desiccated thyroid, 32 mg. daily, and this was progressively increased to 160 mg. daily. Two pellets of desoxycorticosterone acetate, 125 mg. each, and two pellets of testosterone, 150 mg. each, were implanted subcutaneously. The patient was much improved by this treatment as far as her general condition was concerned, but no improvement in her anemia occurred, and she was discharged from the hospital in May 1942.

In November 1943 she entered the Boston City Hospital for replenishment of her therapy. She was given ferrous sulfate by mouth and desoxycorticosterone acetate and testosterone propionate by intramuscular injection. Therapy with desiccated thyroid was continued. Unfortunately, after a month in the hospital she developed virus pneumonia and died after a brief stormy course.

At postmortem examination the pituitary was very small and fibrotic. Microscopic study showed only a few acidophilic, basophilic and chromophobe cells scattered in dense scar tissue. The thyroid gland was one half normal size and the acini were very atrophic. Extensive adrenal atrophy and fibrosis was found. Microscopic examination of the bone marrow was interpreted as showing less than normal erythropoiesis. There was no evidence of a defect in maturation. Cells of the granulocytic series appeared to be normal except for a questionable increase in the eosinophilic series. Megacaryocytes were of the usual number.

*Comment.* The etiology of the pituitary fibrosis in this case remains obscure. The onset of symptoms following influenza suggests that this may have been the etiologic factor. The anemia in this patient was normochromic and normocytic and remarkably constant in severity. It was unaffected by raw liver by mouth, liver by injection, iron in several forms and by hormonal replacement treatment.

*Postpartum necrosis of the pituitary associated with a moderately severe normocytic and normochromic anemia with improvement after hormone therapy.*

*Case 3.* H. O. N. (Details of this case have been reported elsewhere<sup>79-80</sup>) A housewife, aged 42, was admitted to the Boston City Hospital in May 1941 with the signs and symptoms of myxedema. Twelve years prior to entry she had had a severe hemorrhage due to a placenta previa. Following this episode menstruation never recurred and symptoms of hypometabolism were noted. She was treated by several local physicians for hypothyroidism and anemia but the details are not available. She took desiccated thyroid only at irregular intervals and at the time of entry the classic features of myxedema were present, with marked mental and physical retardation. However, her nutritional status was good. Pubic and axillary hair was absent. A hypotension of 90 mm. Hg systolic and 50 diastolic was present. The laboratory findings confirmed the diagnosis of Simmonds' disease. The basal metabolic rate was -43 per cent. Plasma protein bound iodine was less than 1 microgram per cent. Marked sensitivity and hypoglycemic unresponsiveness was demonstrated by an insulin tolerance test (0.04 units of insulin intravenously per kilogram of body weight). No follicle stimulating hormone was found in her urine when tested for 10 rat units per twenty-four hours. Free acid was not present in the gastric juice even after the administration of histamine.

*Hematologic data.* The red blood cell count was 3.45 million, the hemoglobin was 71 per cent and the hematocrit was 30 per cent. Calculation of the blood indices showed a mean corpuscular volume of 87 cu. microns, a mean corpuscular hemoglobin concentration of 37 per cent and a mean corpuscular hemoglobin of 32 micro-micrograms. Two-tenths of one per cent of all erythrocytes were reticulocytes. The white blood cell count was 2,100. The differential count was as follows: polymorphonuclear neutrophils 52 per cent, band forms 14 per cent, eosinophils 2 per cent, basophils 4.5 per cent, lymphocytes 19 per cent, young lymphocytes 1 per cent, monocytes 7.5 per cent.

The patient received several pituitary hormones without clinical or hematologic improvement. There was a decline of the red blood cell count and hemoglobin concentrations as shown in Figure 5. Replacement therapy with nonpituitary hormones was begun on December 29, 1941, with the admini-

tration of methyl testosterone, 30 mg a day by mouth, desiccated thyroid 30 mg a day by mouth and desoxycorticosterone acetate 2 mg a day by intramuscular injection. At a later date pellets of testosterone and desoxycorticosterone acetate were implanted subcutaneously. Details of therapy are indicated in figure 5.

There was a remarkable improvement in the patient's general condition on this therapy. Prior to treatment, the basal metabolic rate was -53 per cent and it rose to normal after treatment for 1 month. The myxedematous changes disappeared and there was a progressive improvement in her energy and strength. The red blood cell count and hemoglobin concentration on January 17, 1942 showed even lower values than previously. The mean corpuscular volume on this occasion was 111 cu microns. One month later the red blood cell count had increased to 4.05 million, with a decrease in the mean corpuscular volume to normal size, 83 cu microns, and 4.5 per cent of the erythrocytes were reticulated. On March

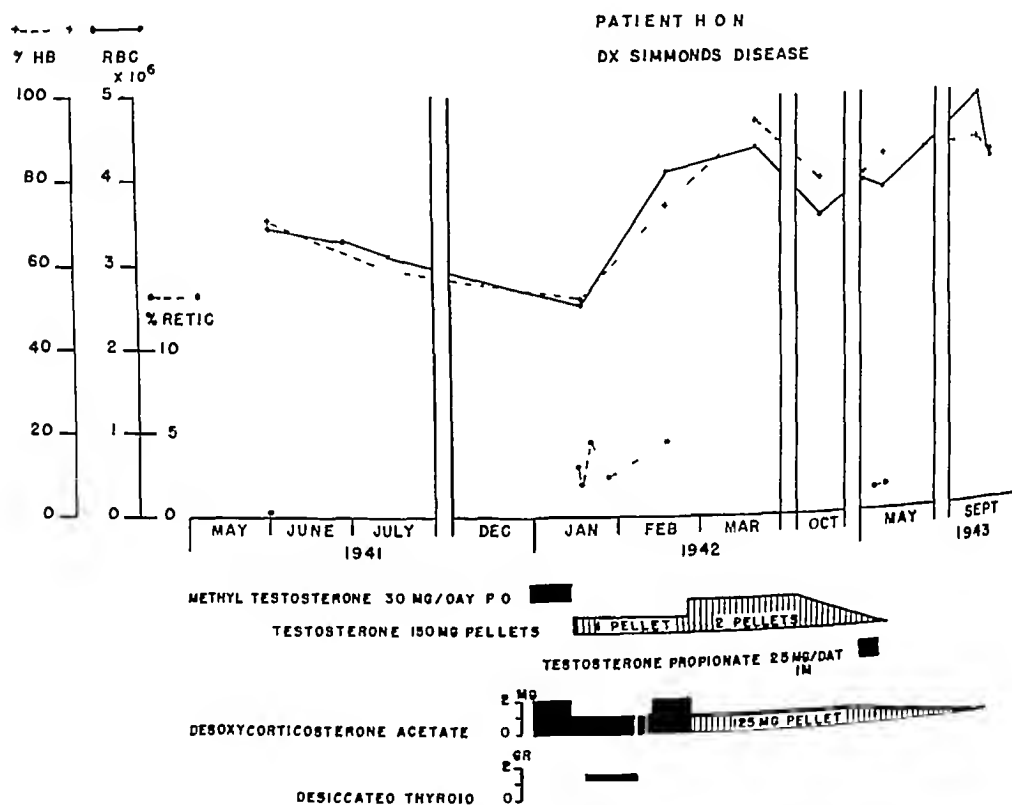


FIG 5 CHANGES IN RED BLOOD CELL COUNT AND HEMOGLOBIN CONCENTRATION FOLLOWING ENDOCRINE THERAPY IN CASE 3

20, 1942 the red blood cell count and hemoglobin had returned to normal levels. On follow up examination the improvement in the blood picture was largely sustained, despite the fact that additional pellets of testosterone could not be procured for replacement.

The patient re entered the Boston City Hospital in January 1944 with pneumonia and, in spite of treatment with sulfathiazole, adrenal cortical extract, desoxycorticosterone and testosterone propionate, she died four days after admission.

At postmortem examination the pituitary was found to be very small and appeared on microscopic examination to be composed largely of fibrous tissue, with rare nests of basophilic cells. Marked atrophy of the thyroid was present and no tissue recognizable as adrenals was found. On microscopic examination the vertebral marrow appeared hypoplastic. Red blood cell and white blood cell precursors were present and showed no evidence of a defect in maturation.

*Comment* The onset of symptoms in this woman following a severe hemorrhage complicating a placenta previa strongly suggests that the atrophy and fibrosis of the pituitary observed at autopsy was due to postpartum necrosis. A moderately severe normocytic anemia was present on admission and became more severe during the period of observation. The macrocytosis observed in January 1942 may have been related to an increase in the severity of her thyroid deficiency as manifested by a basal metabolic rate of  $-53$  per cent in December 1941. On combined hormonal treatment the red blood cell count and hemoglobin concentration returned almost to normal in the course of about three months. It seems likely that testosterone propionate was the agent causing the improvement in the blood picture.

#### SUMMARY AND CONCLUSIONS

Evidence has been presented that the gonads, thyroid, adrenal cortex and pituitary glands have a definite influence on blood formation. The normal sex difference in erythrocyte levels in animals, and probably in man, can be obliterated by castration and restored by appropriate replacement therapy. Hypothyroidism results in a moderately severe anemia in animals. In the uncomplicated form, the anemia is slightly macrocytic and associated with a hypoplastic bone marrow. In clinical experience the anemia may be complicated by the secondary effects of achylia gastrica leading either to iron deficiency or to a deficiency in the anti-pernicious anemia factor. Hyperthyroidism causes some alterations in the white blood cells, but has little effect on the red blood cell series. Hyperactive states of the adrenal cortex may be associated with a mild polycythemia. Adrenal steroids also have a marked lymphocytic effect, causing the release of beta and gamma globulins from lymphoid tissue. A mechanism involving the anterior pituitary and adrenal seems to exist, controlling the release of antibodies under certain conditions. It is suggested that other mechanisms also exist which control the number of circulating lymphocytes.

Deficiency of the anterior pituitary secretions results in anemia in animals and man. The anemia in animals is usually microcytic and hypochromic and may respond to several types of replacement therapy. In man anemia develops slowly and is rarely severe. Moderate reductions in the red blood cell count occur and the color index varies. There is hypoplasia of the bone marrow. The anemia in man does not respond uniformly well to the therapy now available, but improvement often occurs with the replacement of 'end-organ' hormones.

The preponderance of evidence indicates that the regulation of blood formation is not primarily under hormonal control. The effects noted in various glandular disorders are due to alterations in metabolism produced in the bone marrow as well as all other body tissues.

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## OBSERVATIONS ON THE MECHANISM OF HEMOLYSIS IN PAROXYSMAL (COLD) HEMOGLOBINURIA

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**P**AROXYSMAL (cold) hemoglobinuria is a condition observed usually in syphilitics and characterized by transient hemoglobinemia and hemoglobinuria following exposure to cold. Such episodes of hemolysis may be associated with fever, chills, headache, general malaise, abdominal cramps, nausea, vomiting, hives, and Raynaud's phenomena. The most common complaint is dark or bloody urine following exposure to cold.

As late as 1868 this condition had not been differentiated from the hemoglobinuria of malaria.<sup>1</sup> However, by the end of the nineteenth century all of the clinical features mentioned above were recognized and the condition established as a clinical entity.<sup>2</sup> From 1909 to 1929, occasional observations suggested that factors other than cold might activate this hemolytic system. For example, van den Bergh<sup>3</sup> and Hannema and Rytma<sup>4</sup> observed that carbon dioxide under specified conditions in vitro might affect the degree of hemolysis. Manneberg and Donath<sup>5</sup> reported the same results but also observed that carbon dioxide caused hemolysis in normal human blood. Kumagai and Ito<sup>6</sup> reported equivocal results. Although Mackenzie could not duplicate van den Bergh's results in vitro, he observed attacks of hemoglobinuria in a case following emotional disturbances without exposure to changes in temperature.<sup>7</sup> Both van den Bergh and Mackenzie concluded that factors other than cold might activate this system.

Some observers state that serum complement must be present during the cold phase of the Donath-Landsteiner reaction for the hemolysin to be active.<sup>8, 10</sup> Other investigators have reported that complement need be present only during the warm phase.<sup>11, 12</sup> Mackenzie has stated that, although complement may not be essential for the first phase of the Donath-Landsteiner reaction, it is adsorbed during the chilling process when present.<sup>12</sup> In some of his experiments the hemolysin apparently was fixed on the erythrocyte in the absence of complement, but supplementary observations suggested that the presence of complement during chilling increased the degree of hemolysis occurring subsequently at body temperature. Differences in the thermolability have been reported<sup>11</sup> for the hemolysins studied in various cases.

In this communication, observations are reported in an attempt to re-evaluate several of these reported discrepancies. Two cases of paroxysmal (cold) hemoglobinuria were studied, as described briefly in the appendix of this report. The experiments are divided into two groups:

1. The first deals with the fixation of the hemolysin and complement on the

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erythrocytes This group of experiments shows that the hemolysin and at least one component of complement are fixed to the red cells in the cold, and that some thermolabile component of complement must be present in the warm phase of the Donath-Landsteiner reaction for the hemolysin to be effective

2. The second demonstrates that although carbon dioxide, under specified conditions in vitro affected the hemolytic system in the serum of one patient, it failed to do so in the serum from another patient Furthermore, certain substances prevented the hemolytic activity in the serum from one case, but failed to do so in the serum from a second case The effectiveness of these inhibitors did not depend on prevention of fixation of hemolysin on the red cell in the cold phase of the Donath-Landsteiner reaction

### MATERIALS AND METHODS

Group O erythrocytes were washed three times and resuspended in normal saline in a concentration of 5 per cent by volume Guinea pig serum diluted 1 to 5 with normal saline was employed as complement The complement titer\* was recorded as the highest final dilution of serum causing hemolysis in a system containing 0.1 cc aliquots of a 1.5 per cent suspension of sheep cells sensitized with rabbit amboceptor to which serial dilutions of 0.1 cc of serum were added Serum was obtained from blood drawn from the patients into syringes and tubes warmed to body temperature Standard technics were employed for determinations of carbon dioxide, cyanide, and sulfanilamide<sup>13-15</sup> Hydrogen ion determinations were made with a glass electrode employing a Beckman meter The Donath-Landsteiner test was done according to a standard technic<sup>16</sup> The titer of cold hemolysin in the serum was determined by employing serially diluted serum, and a constant amount of complement, in the Donath-Landsteiner test The anti human serum rabbit serum was prepared and used as described by Coombs et al<sup>17</sup>

### EXPERIMENTAL RESULTS

#### Group I *Experiments on the fixation of hemolysin and complement during the Donath-Landsteiner reaction*

The object of the following experiment was to determine the phase of the Donath-Landsteiner reaction during which complement and hemolysin were adsorbed by the erythrocyte

##### *A Experiment on fixation of hemolysin and complement in the cold*

Washed normal Group O red blood cells were packed in 0.5 cc aliquots in four tubes (see table 1) To two of the tubes (Nos 1 and 3) 2 cc of serum from Case 1 were added To the other two tubes (Nos 2 and 4) 2 cc of serum from Case 2 were added Then 0.5 cc of saline was added to all tubes Tubes 1 and 2 were kept at 27 C for one hour with frequent mixing Tubes 3 and 4 were chilled at 2 C for thirty minutes and then centrifuged after being packed with ice in large centrifuge metal cups Tubes 1 and 2 were centrifuged at 27 C The clear supernatant was removed quickly from all four tubes

*Results and conclusions* The titer of complement and hemolysin in the supernatant removed from the cell-serum mixtures kept at 27 C were both high However, both hemolysin and complement activity disappeared from the serum previously chilled at 2 C in the presence of cells Presumably, hemolysin and one or more components

\* The term complement activity is used to indicate the hemolytic reaction of serum with sensitized sheep cells as described above Since all components of complement are necessary for the hemolytic reaction with sensitized sheep cells, the absence of complement activity is interpreted as indicating the lack of one or more of these components

of complement were adsorbed by the red blood cells at the lower temperature but not at the higher one. Such adsorption of hemolysin and complement from the serum of Case 2 caused no significant alteration in the electrophoretic pattern of the serum.<sup>18</sup>

The question then arose as to what would occur if hemolysin and complement were separated and chilled separately in the presence of red blood cells.

*B Experiment on complement and hemolysin activity in the cold phase of the Donath-Landsteiner reaction*

Serum from Case 2\* was heated at 63 C for three minutes. Two-tenths of a cubic centimeter of this serum caused no hemolysis of 0.2 cc of a 1:5 per cent suspension of sensitized sheep cells (see tube 1 of table 2). The remainder of the serum was divided into 0.5 cc aliquots and, as shown in table 2, was placed in tubes 3 and 5. Five-tenths of a cc of unheated serum were placed in two other tubes (see tubes 2

TABLE 1—Adsorption of Hemolysin and Complement

Test tube	Case 1 serum	Case 2 serum	Control packed R B C	Normal saline	Hemolysin in* supernatant	Complement in† supernatant
	cc	cc	cc	cc	titer	titer
1	2.0		0.5	0.5	1/4	1/128
2		2.0	0.5	0.5	1/32	1/32
3	2.0		0.5	0.5	0	0
4		2.0	0.5	0.5	0	0

Procedure: Test tubes 1 and 2 left at room temperature (27 C) for 1 hour, then centrifuged at 27 C, and supernatant tested for hemolysin and complement. Test tubes 3 and 4 chilled at 2 C for 30 mins with frequent stirring, then centrifuged in cold, and supernatant tested for hemolysin and complement.

\* As determined by Donath-Landsteiner tests on serially diluted supernatant.

† As determined by adding 0.1 cc 1:5% sensitized sheep R B C to 0.1 cc portions of serially diluted supernatant.

and 4 of table 2.) To the four tubes were added 0.2 cc of a 5 per cent suspension of washed normal Group O red blood cells. One-tenth cc of normal saline was added to all four tubes. To tubes 2 and 3 were added 0.2 cc of a 1:5 dilution of guinea pig serum. All four tubes were then chilled at 2 C for ten minutes. After the chilling, 0.2 cc of a 1:5 dilution of guinea pig serum were added to tubes 4 and 5. All tubes were then incubated at 37 C for 1 hour.

*Results and conclusions* As shown in table 2, hemolysis did not occur if cells were chilled in heat-inactivated serum unless complement was added *before* chilling. Adding complement to previously heated serum *after* the chilling phase of the Donath-Landsteiner reaction did not cause hemolysis. Apparently, complement had to be present during the cold phase of the reaction to cause hemolysis. This was also suggested by rechilling tube 5 of table 2. Such subsequent rechilling and re-warming resulted in almost complete hemolysis. This substantiated the conclusion

\* The hemolysin in the serum of Case 1 was found to be more thermolabile than complement when heated at 56 C. Attempts to separate the thermolabile hemolysin and the complement by adsorption on other antigen-antibody precipitates failed since both were adsorbed simultaneously. In contrast, the hemolysin in the serum of Case 2 was thermostable, thus allowing inactivation of the complement without destroying the hemolysin.

that hemolysin was present but was active only when chilled in the presence of complement

These conclusions were substantiated by another technic. The serum of Case 2 and guinea pig serum were heat inactivated at 57 C for thirty minutes. These sera and unheated sera were combined as shown in table 2-A and chilled with cells at 3 C for ten minutes, then incubated at 37 C for thirty minutes. Small aliquots of cells chilled in the presence of the various combinations of sera shown in table 2-A

TABLE 2 —Effect of Complement on the Cold Phase of the Donath-Landsteiner Reaction

Test tube	Case 2 serum	Case 2 serum heated at 63 C-3min	Cont R B C	Compl added before chilling	Compl added after chilling	Norm saline	Sensitized sheep R B C (1.5%)	Hemolysis
1	cc	cc	cc	cc	cc	cc	cc	0
2	0.5	0.2	0.2	0.2		0.1	0.2	++++
3		0.5	0.2	0.2		0.1		+++
4	0.5		0.2		0.2	0.1		++++
5		0.5	0.2		0.2	0.1		0

Procedure: All tubes chilled at 2 C for 10 minutes, then incubated at 37 C for one hour

TABLE 2 a —Demonstration of Adsorption of the Hemolysin on Red Cells by the Agglutination of such Cells (in a Saline Suspension) following the Addition of Anti-human Serum Rabbit Serum

Test tube	Case 2 serum	Case 2 serum heated at 57 C 30 min	Complement	Complement heated at 57° C 30 min	R B C (5 per cent suspension)	Hemolysis*	Coomb's serum agglutination†
1	cc	cc	cc	cc	cc		
2	0.5		0.2		0.3	2+	2+
3		0.5	0.2		0.3	tr	1+
4	0.5	0.5		0.2	0.3	0	0
5				0.2	0.3	1+	2+

\* Hemolysis as determined after chilling the suspension 10 minutes and incubating at 37 C for 1 hour

† Coomb's test was performed by incubating 0.2 cc of a cell suspension with 0.2 cc Coomb's serum and expressing the agglutination as 4+ to 0 on microscopic examination

were removed prior to incubation at 37 C and resuspended in several cc of saline. By resuspending the cells in saline immediately after chilling, they could be kept indefinitely without lysis (see tubes 3 and 6 of table 3). Two-tenths of a cubic centimeter of such suspensions of red cells were then incubated at 37 C for one hour with Coomb's serum.<sup>17</sup>

Hemolysis occurred only when the serum of Case 2 or that of the guinea pig had not been previously heated. Heating both sera prior to the chilling phase of the Donath-Landsteiner test prevented subsequent hemolysis. Cells, after being chilled in the presence of both hemolysin and intact complement and resuspended in saline, were agglutinated by anti-human serum rabbit serum.<sup>17</sup>

This agglutinability of the cells occurred following their chilling in the presence of the hemolysin when guinea pig serum furnished the only complement, it did not occur when both the complement in the patient's and guinea pig sera had been previously heat inactivated. These observations suggested the hemolysin was adsorbed only in the presence of intact complement, whether the latter was furnished by the human serum or guinea pig serum.

Since both the hemolysin and at least the thermolabile components of complement were required in the cold to sensitize red cells, the role of complement during the warm phase of the Donath-Landsteiner reaction was studied. Therefore, the following experiment was performed.

TABLE 3 — *Effect of Complement on the Warm Phase of the Donath-Landsteiner Reaction*

Test tube	Sensitized* cont R B C (5%)		Compl	Compl heated at 63 C -3 min	Normal saline	Hemolysis
	Case 1	Case 2				
	cc	cc	cc	cc	cc	
1	0.2		0.2			++++
2	0.2			0.2		0
3	0.2				0.2	0
4		0.2	0.2			++++
5		0.2		0.2		0
6		0.2			0.2	0

Procedure: Compl, heated compl, and normal saline added to sensitized cont R B C at room temperature (circum 27 C). All tubes then incubated at 37 C for one hour.

\* Sensitized cont R B C prepared by chilling cont R B C for 15 mins at 2 C in presence of sera of case 1 and case 2. R B C then washed with normal saline and resuspended in 5% concentration.

### C. *Experiment on complement and hemolysin activity in the warm phase of the Donath-Landsteiner reaction*

Packed washed normal Group O red cells were chilled at 2 C for fifteen minutes in twice their volume of unheated serum from Case 1 or Case 2 (see table 3). Saline, chilled to 4 C, was then added and the cells washed while cold. They were then resuspended in normal saline in 5 per cent concentration and warmed to 27 C. Two tenths of a cubic centimeter of the suspension of red cells previously chilled in the serum of Case 1 were placed in three tubes (tubes 1, 2, and 3 of table 3) and equal aliquots of cells previously chilled in the serum of Case 2 were placed in three other tubes (tubes 4, 5, and 6). Two-tenths of a cubic centimeter of guinea pig serum diluted 1 to 5 were added to tubes 1 and 4, 0.2 cc of guinea pig serum diluted 1 to 5 with saline and inactivated by heating at 63 C for three minutes were added to tubes 2 and 5, and 0.2 cc of saline were added to tubes 3 and 6. All the tubes were then incubated at 37 C for one hour.

**Results and conclusions** Cells that were previously chilled in the presence of fresh serum from both cases, then washed and resuspended in saline, did not hemolyze on incubation unless intact complement was present also during the warm phase. Adding heat inactivated complement or more saline did not cause hemolysis. Presumably, red cells sensitized in the cold phase by hemolysin and complement could not be hemolyzed in the warm phase unless intact complement was present. Thus,

some thermolabile component of complement was required during the warm phase of the Donath-Landsteiner reaction

*Group II Experiments with carbon dioxide and inhibitors of carbonic anhydrase*

As previously reported,<sup>19</sup> relatively high contents of carbon dioxide, under certain conditions in vitro, may cause hemolysis of cells suspended in the serum of some cases of paroxysmal (cold) hemoglobinuria. Other observers,<sup>6, 2</sup> in similar experiments, have not obtained these results. In a previous communication,<sup>19</sup> preliminary observations were made on the effect of carbon dioxide on the Donath-Landsteiner reaction of Case 1, as reported in more detail here. One possible interpretation for the discrepancy in the reports concerning the effect of carbon dioxide might be that there were possible differences in the temperatures at which the experiments were performed by different observers. For example, it had been reported<sup>19</sup> that carbon dioxide caused complete hemolysis of Group O cells suspended in the patient's serum (Case 1) when the temperature was 27 C, but no hemolysis occurred at 37 C. It was conceivable, therefore, that exposure to relatively high temperatures had resulted in no hemolysis, whereas lower temperatures could have produced hemolysis. It had already been established that the hemolysin in the serum of various patients was activated at different temperatures.<sup>2</sup> Accordingly, there was a distinct possibility that the effect of carbon dioxide on the hemolytic system in the sera of various cases might appear at different temperatures. However, further observations suggested that a simple difference in temperature threshold was probably not the explanation for such discrepancies in the literature. For example, bubbling in carbon dioxide under oil at a temperature of 11 C caused no more hemolysis of cells suspended in the serum of Case 2 than allowing such suspensions to simply stand at the same temperature for the same length of time. Furthermore, cyanide and sulfanilamide, when employed as reported before,<sup>19</sup> did not inhibit the hemolytic activity of the serum from Case 2. These results were in striking contrast to those observed in the study of Case 1.<sup>19</sup> Accordingly, the effect of sulfanilamide and cyanide on the Donath-Landsteiner reaction was studied in more detail by using the serum from Case 1, as indicated in the following series of observations.

*A Experiments on the phase of action of sodium sulfanilamide and cyanide on the Donath-Landsteiner reaction*

Two-tenths of a cubic centimeter of a 5 per cent suspension of normal washed Group O red blood cells were placed in each of 6 tubes (see table 4). To tube 1 was added 0.1 cc. of normal saline, to tubes 2, 3, 5, and 6 were added 0.1 cc. of complement as guinea pig serum diluted 1 to 5 with saline. (As complement was already present in the serum of Case 1 [see table 1] the addition of more complement was actually unnecessary.) One-tenth of a cubic centimeter of 0.08 M solutions of sodium cyanide and sodium sulfanilamide were added to tubes 2 and 3, respectively. Five-tenths of a cubic centimeter of serum from Case 1 were placed in all tubes. All tubes were then chilled at 2 C for fifteen minutes. At the end of that time, 0.1 cc. of normal saline, 0.08 M sodium cyanide, and 0.08 M sodium sulfanilamide were added to tubes 4, 5, and 6, respectively. All tubes were then incubated at 37 C for one hour.

*Results and conclusions* Hemolysis occurred in all tubes except those chilled in the presence of sulfanilamide and cyanide. Adding sulfanilamide and cyanide after

the chilling phase did not prevent hemolysis. Apparently, therefore, cyanide and sulfanilamide had to be present during the chilling phase of the Donath-Landsteiner reaction in order to prevent hemolysis.

These observations raised the question of whether these substances prevented sensitization of red cells by the hemolysin or prevented the hemolysis of already sensitized cells. The following experiment was performed in the attempt to answer this question.

*B Experiment on the effect of sodium sulfanilamide and cyanide on the union of hemolysin with red blood cells*

Four-tenths of a cubic centimeter of a 5 per cent suspension of normal washed Group O cells were placed in each of 3 tubes (see table 5). Four-tenths of a cubic centimeter of complement in the form of guinea pig serum diluted with 1 to 5 with saline and 1.0 cc. of serum from Case 1 were added. Two tenths of a cubic centimeter of normal saline, 0.08 M sodium cyanide, and 0.08 M sodium sulfanilamide were added to tubes 1, 2, and 3, respectively. The tubes were then chilled at 2 C for twenty minutes, centri-

TABLE 4—Observations on the Time of Action of Inhibitors on the Donath-Landsteiner Reaction

Test tube	Case 1 serum	Control R B C	Compl	Normal saline	Sodium cyanide	Sodium sulfanil amide	Hemolysis
	cc	cc	cc	cc	cc	cc	cc
1	0.5	0.2		0.1			+++
2	0.5	0.2	0.2		0.1		0
3	0.5	0.2	0.2			0.1	0
4	0.5	0.2		0.1			+++
5	0.5	0.2	0.2		0.1		+++
6	0.5	0.2	0.2			0.1	+++

Procedure: Test tubes 1 and 4 are controls. 0.1 cc. of saline, sodium cyanide, sodium sulfanilamide added to test tubes 1, 2, and 3 before chilling at 2 C for 15 minutes. Same reagents added to test tubes 4, 5, and 6 after chilling at 2 C for 15 minutes. All tubes then incubated at 37 C for one hour.

fuged in the cold by packing the tubes in ice and the supernatant pipetted off. The cells were then washed in cold phosphate buffer (see table 5). The cells of tubes 2 and 3 were washed in cold phosphate buffer containing 0.008 M concentration of sodium cyanide and sodium sulfanilamide respectively. 1 cc., in a known effective concentration of inhibitors to prevent any union of hemolysin with the red cells during the washing in the chilled state that might occur as the concentration of inhibitors decreased. The phosphate buffer at pH 7.2 was used to prevent the alkalizing effect of sodium cyanide and sodium sulfanilamide in unbuffered solution. Such a high pH might inactivate complement or elute the hemolysin from the red cell. After washing twice in the above solutions, the cells were resuspended in normal saline and the cell suspensions were then used as shown in table 6. The cell suspensions from each tube represented in table 5 were warmed to 27 C and divided into aliquots of 0.2 cc. each. To tubes 1, 3, and 5 were added 0.2 cc. of normal saline and to tubes 2, 4, and 6 were added 0.2 cc. of complement in the form of guinea pig serum diluted 1 to 5 with saline. The tubes were then incubated at 37 C for one hour.

*Results and conclusions* Hemolysis occurred as shown in table 6. This indicated that the effect of sulfanilamide and cyanide on the cells and serum was a reversible one, and also that the presence of sulfanilamide and cyanide in the cold did not prevent the union of the hemolysin with the red cell. The red cells had apparently

become sensitized by the hemolysin in the cold in spite of the presence of the inhibitors. The subsequent withdrawal of the inhibitors allowed the hemolysis to proceed during the incubation at 37 C.

These conclusions were substantiated by the following experiment.

C *Experiment on the effect of sodium cyanide on the union of hemolysin with red blood cells*

TABLE 5—*Observations on Effect of Inhibitors on Union of H. molysin and Complement with R B C (Part A)*

Test tube	Case 1 serum	Control R B C	Compl	Normal saline	Sodium cyanide (0.8 M)	Sodium sulfanilamide (0.8 M)
	cc	cc	cc	cc	cc	cc
1	1 0	0 4	0 4	0 2		
2	1 0	0 4	0 4		0 2	
3	1 0	0 4	0 4			0 2

Procedure: Tubes chilled at 2 C for 20 mins, centrifuged in cold, and supernatant pipetted off.

Test tube 1—R B C washed twice with 5 cc isotonic phosphate buffer (pH 7.2).

Test tube 2—R B C washed twice with 5 cc isotonic phosphate buffer (pH 7.2) containing 0.08 M sodium cyanide.

Test tube 3—R B C washed twice with 5 cc isotonic phosphate buffer (pH 7.2) containing 0.08 M sodium sulfanilamide.

R B C of each tube then resuspended in 0.4 cc norm saline and employed as shown in next table (part B).

TABLE 6—*Observations on Effect of Inhibitors on Union of Hemolysin and Complement with R B C (Part B)*

Test tube	R B C suspensions in saline as prepared in part (A)			Normal saline	Compl	Hemolysis
	From T T 1	From T T 2	From T T 3			
	cc	cc	cc	cc	cc	
1	0 2			0 2		0
2	0 2				0 2	+++
3		0 2		0 2		0
4		0 2			0 2	++
5			0 2	0 2		0
6			0 2		0 2	++

Procedure: Compl and normal saline added to test tubes at room temp (27 C). All tubes then incubated at 37 C for one hour.

To 1 cc of packed Group O cells in three separate test tubes were added 0.5 cc of a buffered 0.08 M sodium cyanide solution (tubes 1, 2, and 4). To another tube (tube 3) of 1 cc of packed red cells, 0.5 cc of saline was added. To each tube were then added 0.2 cc of guinea pig serum and 3.3 cc of the serum of Case 1. One tube containing cyanide (tube 1) was allowed to stand at 34 C. The other three tubes were chilled at 3 C for thirty minutes. Two of the chilled tubes (tube 2 containing cyanide, and tube 3 not containing cyanide) were then centrifuged in chilled containers with packed ice. The contents of the tube (tube 1 containing cyanide) not chilled, were centrifuged at room temperature. The supernatant in the centrifuged tubes were removed and the cells resuspended in 200 cc of saline. The fourth tube (tube 4) contained cyanide, and as a control was incubated at 37 C for one hour following chilling. The supernatant from the tubes 1, 2, and 3, were then dialyzed in cellophane bags against normal saline overnight.

at icebox temperature. The dialyzed supernatants were then used in Donath Landsteiner reactions (see table 7) and titrated for complement. Small aliquots of the cell suspensions were incubated for one hour at 37 C in equal aliquots of Coomb's serum and with complement (see table 8).

**Results and conclusions** Cells chilled in the serum of Case 1 both in the presence and absence of cyanide were agglutinated in the Coomb's test and showed hemol-

TABLE 7—*Observations Showing Cyanide did not Prevent the Union of Hemolysin with the Red Cell during Chilling*

Test tube	Dialysed supernatant serum from			Complement	Saline	R B C (5 per cent suspension)	Hemolysis*
	Tube 1	Tube 2	Tube 3				
	cc	cc	cc	cc	cc	cc	
1	0 5			0 2	0 1	0 2	4+
2		0 5		0 2	0 1	0 2	0
3			0 5	0 2	0 1	0 2	1+

Tube 1 = serum previously incubated at 34 C for 30 minutes with cyanide and red cells (see text), dialyzed and employed as shown

Tube 2 = serum previously chilled at 3 C for 30 minutes with cyanide and red cells (see text), dialyzed and employed as shown

Tube 3 = serum previously chilled at 3 C for 30 minutes with red cells but in the absence of cyanide (see text), dialyzed and employed as shown

\* Hemolysis as determined after chilling the suspension at 2 C for 10 minutes and incubating at 37 C for 1 hour

TABLE 8—*Observations Showing the Adsorption of Hemolysin by Red Cells during Chilling in both the Presence and Absence of Cyanide*

Test tube	Washed cells from			Coomb's serum	Complement	Agglutination*	Hemolysis*
	Tube 1	Tube 2	Tube 3				
	cc	cc	cc	cc	cc		
1	0 1			0 1		0	Neg
2	0 2				0 2		
3		0 1		0 1		2+	Pos
4		0 2			0 2		
5			0 1	0 1		2+	Pos
6			0 2		0 2		

Tube 1 = cells previously incubated at 34 C for 30 minutes with cyanide and hemolysin, washed in saline and employed as shown

Tube 2 = cells previously chilled at 3 C for 30 minutes with cyanide and hemolysin, washed in saline and employed as shown

Tube 3 = cells previously chilled at 3 C for 30 minutes with hemolysin but without cyanide, washed in saline and employed as shown

\* Agglutination and hemolysis as determined after 1 hour incubation at 37 C

ysis on incubation with complement at 37 C after being washed in a large amount of saline (table 8). Employing the dialyzed supernatant from the chilled tubes in the Donath-Landsteiner reaction showed that the hemolysin was absent or present in only a trace. That the hemolysin and complement had been adsorbed and not



simply destroyed by cyanide was shown by the marked hemolytic activity in a Donath-Landsteiner reaction of the dialyzed supernatant from the tubes allowed to stand at 34 C for thirty minutes. Complement was present in that supernatant in a titer of 16 units per cc. That the original cyanide concentration was an effective one was shown by the slight trace of hemolysis in an identical suspension (tube 4) chilled for the same period of time and incubated at 37 C for one hour. Thus, by a second technic, adsorption of hemolysin and complement by the red cell in the presence of cyanide was demonstrated.

### DISCUSSION

Both hemolysin and some component of complement were adsorbed by red blood cells from the sera of each of 2 cases in the cold phase of the Donath-Landsteiner reaction. This observation supports some of those reported by Cooke,<sup>20</sup> Hoover and Stone,<sup>8</sup> and by Dennie and Robertson.<sup>9</sup> Cooke<sup>20</sup> has pointed out that although Donath and Landsteiner<sup>21</sup> maintained the view that cold was necessary only for the union of red blood cell and hemolysin and that complement united in the warm phase, their experiments did not actually prove this. Moss<sup>22</sup> concluded that complement did not enter into the reaction of the cold phase (at least not permanently). His evidence consisted of the demonstration of complement in the supernatant serum after chilling with cells. This does not rule out the possibility that some complement was actually used. Hoover and Stone<sup>8</sup> reported that after chilling red cells in heat-inactivated serum from a patient with paroxysmal cold hemoglobinuria, incubating then in normal serum at 37 C did not cause hemolysis. Washing cells in saline and rechilling and reincubating them in normal saline did cause hemolysis. They interpreted their results as showing a complement factor must also be adsorbed in the cold by cells before they are susceptible to hemolysis on subsequent incubation. Hemolysin and complement in the serum of Case 2 were separable by heat. After inactivating the serum from Case 2 for complement, the hemolysin remained. Chilling and incubating cells in such inactivated serum did not cause hemolysis. Nor did restoring complement to the previously inactivated serum-cell suspension after chilling cause hemolysis. However, rechilling and reincubating at 37 C such a suspension following the addition of complement did cause hemolysis, indicating complement had to be present during the cold phase of the Donath-Landsteiner reaction for the hemolysin to be effective. Therefore, the assumption that complement acts only in the warm phase of the Donath-Landsteiner reaction under these defined *in vitro* conditions is incorrect in at least one instance.

Dennie and Robertson<sup>9</sup> observed that complement was necessary not only in the cold but also for the warm phase of the Donath-Landsteiner reaction. Although Cooke<sup>20</sup> concluded from his work that complement united solely in the cold, some of his observations show that complement was a requisite for the warm phase also. Throughout several of his experiments, cells were chilled in the patient's serum and then resuspended in saline. Cooke reported no hemolysis in such saline suspensions on rewarming. However, if both complement and hemolysin were active only in the cold, then rewarming such saline suspensions should result in hemolysis. Chilling cells in the presence of complement and hemolysin and resuspending them

in either saline or heat-inactivated complement was followed by no hemolysis on incubation at 37 C. Adding intact complement to such warm suspensions, however, did cause hemolysis. Apparently, complement was necessary for the warm as well as the cold phase of the Donath-Landsteiner reaction.

The hemolysin in the serum of Case 1 was inactivated by heating before complement was destroyed. Heating the serum of Case 2 long enough to inactivate the complement did not inactivate the hemolysin. Mackenzie<sup>2</sup> has also observed differences in the thermostability of hemolysins from various patients with this disease entity, and Yorke and Macfie<sup>10</sup> found the thermolability of the hemolysin in the serum of 1 patient actually varied from time to time.

Several observers have shown that carbon dioxide may be an *in vitro* activating factor in this hemolytic system.<sup>3,4</sup> As reported previously, when cells were suspended in the serum of Case 1 at 27 C with carbon dioxide in a concentration of 3.6 mEq per liter (pH 6.4) lysis rapidly occurred.<sup>19</sup> The same procedure at 37 C did not cause hemolysis and lysis did not occur even at 27 C if the serum hemolysin had been previously removed. Furthermore, sulfanilamide and cyanide prevented the lysis of a Donath-Landsteiner reaction when this same serum was employed. As the study of other patients' sera had revealed no such effect of carbon dioxide,<sup>2</sup> it was thought that temperature might have been a critical variable. Thus, workers employing lower temperatures might activate the system with carbon dioxide, while those using higher *in vitro* temperatures might obtain negative results. The upper limit of temperature at which the serum hemolysin becomes effective seems to vary from patient to patient.<sup>2</sup> As the carbon dioxide *in vitro* effect is dependent on the presence of hemolysin,<sup>19</sup> it seemed logical that the carbon dioxide activity at a given temperature might depend on the thermal amplitude of that particular hemolysin. Although such temperature factors may be important, study of Case 2 suggests that they are not the only ones. At a temperature (11 C) which alone activated the system *in vitro*, the use of carbon dioxide did not increase the amount of hemolysis of cells suspended in the serum of Case 2. Mackenzie had previously reported that carbon dioxide did not activate the *in vitro* hemolytic system of sera from several patients with this disease whom he had studied.<sup>2</sup> In contrast to the effectiveness of sulfanilamide and cyanide in preventing hemolysis of cells suspended in the serum of Case 1, these inhibitors were ineffective in the prevention of hemolysis of cells suspended in the serum of Case 2. Thus, the effectiveness and ineffectiveness of carbon dioxide, sulfanilamide, and cyanide ran parallel in the hemolytic system.

Sulfanilamide and cyanide in the serum of Case 1 did not prevent the union of the hemolysin and complement with the red cell, but did prevent the subsequent lysis. The adsorption of the requisite serum factors for lysis by the erythrocytes when chilled in both the presence and absence of such inhibitors was shown in two ways. Hemolysin and complement either decreased markedly in concentration, or actually disappeared from the supernatant of all such chilled suspensions. Furthermore, the cells from such chilled suspensions when washed in cold saline and then incubated at 37 C with fresh complement were lysed. This showed the hemolysin had acted on the cell membrane irrespective of the presence of the inhibitors. Lastly, if cells

chilled in the presence of cyanide and hemolysin were washed in saline and then incubated in Coomb's serum, they were agglutinated. This was further evidence that some serum factor had been adsorbed on the red cell. Such agglutination of the cells by an anti-human serum rabbit serum occurred even though the thermolabile components of complement were furnished by guinea pig serum.

The prevention of lysis was not due to an inactivation of the complement necessary during the warm phase of the Donath-Landsteiner reaction. This was established by the lack of effect of the inhibitors when added to the red cell-serum suspension after chilling but before warming. The inhibitors had to be present during the entire Donath-Landsteiner reaction in order to be effective. This suggests the possibility that these substances may be acting at the red cell membrane, and though not preventing the union of the hemolysin-complement complex with the red cell, may, at least in some cases, prevent some subsequent alteration in the spatial configuration of the hemolysin-complement-red cell membrane complex that is a requisite for actual hemolysis. The differences in the effectiveness of these inhibitors might, therefore, be associated with differences in the size of the antibody involved in various sera.

Apparently, the union in the cold of hemolysin and complement with the red cell membrane does not necessarily lead to hemolysis. Some other step must occur. That the latter may be delayed and yet be effective is indicated by the reversibility of the inhibiting effect of cyanide and sulfanilamide. That the inhibiting activity of these two substances does not depend on preventing either the union of the requisite serum factors in the cold or the activity of some additional factor during the warm phase has been illustrated in three ways: (1) Both hemolysin and complement disappear or, at least, diminish in concentration from the supernatant of a chilled suspension containing either of the two inhibitors. (2) This diminution in titer is due to adsorption and not destruction as illustrated by subsequent positive Coomb's tests as well as lysis of the cells when washed and incubated in complement at 37 C. (3) Finally, the inhibitors do not prevent the lysis unless present before chilling. Their ineffectiveness when added only during the warming phase indicates their action is not due to some effect on a serum factor necessary during that phase of the Donath-Landsteiner test.

Thus, by elimination of several possible interpretations, the question arises whether or not by acting at the cell membrane these substances simply prevent reorientation of the antibody-complement complex on the cell membrane. By this concept, the complex could unite with the red blood cell in the presence of the inhibitors and when the latter are removed, reorientation on the membrane could occur and lysis result. In such a concept two possibilities would be suggested: (1) carbonic anhydrase may be contained in the red blood cell membrane and (2) the differences in the effectiveness of the inhibitors from case to case may depend on the difference in the size of the hemolysin molecule in the two sera. Thus, the more thermolabile and possibly larger hemolysin could not reorient itself on the cell membrane in the presence of the inhibitors but the thermostable and possibly smaller antibody could. As a result, the inhibitors would be effective in the first instance and not in the second.

## CONCLUSIONS

- Observations made on 2 cases of paroxysmal (cold) hemoglobinuria showed
- 1 Hemolysin and complement were adsorbed by erythrocytes in the cold
  - 2 A thermolabile fraction of complement was necessary for both the cold phase and the warm phase of the Donath-Landsteiner reaction in the serum of 1 case
  - 3 Under specified in vitro conditions, carbon dioxide affected the hemolytic system of 1 case and not that of the other, this carbon dioxide effect was itself subject to the influence of temperature
  - 4 Sulfanilamide and cyanide inhibited the Donath-Landsteiner reaction in the serum of 1 case and not in the serum of the other
  - 5 These inhibitors did not prevent the union in the cold of the hemolysin-complement complex, but did prevent the usual effect of such a union in 1 case
  - 6 If not present during the chilling phase, sulfanilamide and cyanide did not prevent hemolysis
  - 7 Under specified conditions, sulfanilamide and cyanide did not prevent the effect of the thermolabile component of complement in the warm phase of the Donath-Landsteiner reaction
  - 8 The differences in the observations of the 2 cases reported here and some apparent discrepancies cited from the literature suggest the possibility of important fundamental variants in the mechanism of the disease

## APPENDIX

*Case Histories*

## CASE 1

W B (J H H, history number 359265), a 33 year old Negro, was admitted with the chief complaint of upset stomach and dark urine after exposure to cold. The family history was noncontributory. The past history revealed that fifteen years prior to admission the patient had had a penile lesion and was given antiluetic treatment for one and one-half years. The patient dated the present illness as beginning three years prior to admission. At that time, he noted attacks of abdominal cramps, nausea, and vomiting following exposure to cold. These episodes were associated with the passage of very dark urine in noticeably small quantities. The attacks lasted for several hours but the patient thought he could shorten their duration by drinking hot coffee or warming himself. The physical examination was essentially negative. The pertinent laboratory data were as follows. The serologic test for syphilis was positive. There was a mild normocytic anemia. The icterus index was 12. The urine was dark brown. There was a 3 plus albuminuria as well as numerous red blood cells and white blood cells per high power field. The serum nonprotein nitrogen was 71 mg per cent. The phenosulfonphthalein excretion was 38 per cent at the end of two hours. Urea clearance was 48 per cent of normal maximum. The Donath-Landsteiner reaction was positive. Cold agglutinins were present in a titer of 1 to 160 and were more thermostable than the cold hemolysin. The clinical diagnosis was syphilis associated with paroxysmal (cold) hemoglobinuria and renal impairment.

## CASE 2

M E (J H H, history number 414118), a 30 year old Negro, was admitted with the chief complaint of dark urine following exposure to cold. The family and past histories were noncontributory. The present illness apparently began two years prior to admission with periodic attacks of swelling of the eyelids, lips, periorbital tissues and fingers on exposure to cold. This swelling was associated with itching of the affected parts. The swelling and itching would disappear usually within twenty minutes when the patient became warm. Such exposure to cold would occasionally be associated with mild attacks of

dominal cramps There was no nausea or vomiting One year prior to admission the patient noticed that her urine became red, dark, and almost black after chilling The urine would remain dark for only four to six hours The patient stated that her eyes had been yellow occasionally during the past winter Drinking ice water would result in a tight sensation in her throat No dark urine had been noted following consumption of cold drink or food The physical examination was essentially negative except for slight pallor of the nail beds and mucous membranes The significant laboratory data were as follows The serologic test for syphilis was positive There was a moderate macrocytic anemia The icterus index was 15 Cold agglutinins were present in a titer of 1 to 320 The urine was dark brown and guaiac positive There were occasional red blood cells and white blood cells per high power field The Donath Landsteiner test was positive The clinical diagnosis was syphilis associated with paroxysmal (cold) hemoglobinuria and angioneurotic edema following exposure to cold

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# THE EVENTS IN THE HEMOLYTIC CRISIS OF HEREDITARY SPHEROCYTOSIS, WITH PARTICULAR REFERENCE TO THE RETICULOCYTOPENIA, PANCYTOPENIA AND AN ABNORMAL SPLENIC MECHANISM

By WILLIAM DAMESHEK, M D AND MARVIN L. BLOOM, M D

**H**EREDITARY spherocytosis (familial hemolytic jaundice), although a chronic disease of varying severity, at times takes on the aspects of an acute illness. The patient, previously in fairly good health, suddenly experiences malaise, vomiting, fever and nausea. Examination reveals an unusual degree of pallor. In severe instances, a varying degree of shock is present. For these acute episodes, the designation of 'hemolytic crisis' has long been used.

The cause of the crisis has not been determined. If the disease is due simply to the production of abnormal red cells, i.e., spherocytes, and their removal by an essentially normal spleen, the crisis would have to be explained by a sudden great increase in maldevelopment of the bone marrow red cells. This is hardly likely. Both Doan<sup>13</sup> and Heilmeyer<sup>20</sup> have postulated that the spleen is largely at fault, becoming unusually active during the crisis. In a previous report of three cases of crisis occurring successively in members of the same family, one of us<sup>6</sup> noted a consistent reduction in leukocytes, platelets and reticulocytes. It was speculated that this might be due to an unusual degree of splenic activity with inhibitory effects upon blood formation in the bone marrow. In a more recent article, Owen<sup>38</sup> emphasized the pancytopenia and particularly the reticulocytopenia. He suggested that the crisis could not be considered as hemolytic but was actually aplastic and stated that no proof of the hemolytic nature of the crisis was available. The possibility that hypersplenic effects might be present was discounted.

Our observations in seven cases of hemolytic crisis have led us to conclude that the crisis is due to the combination of (1) a marked exaggeration of the usual hemolytic mechanism with (2) arrested maturation of red cells in the bone marrow induced by a pathologically hyperactive spleen. The marrow in crisis shows not aplasia but rather maturation arrest of the nucleated red cells at the most primitive or erythrogonic level. The dramatic and almost immediate effects of splenectomy, with the sudden outpouring of red cells, leukocytes and platelets indicate strongly that the marrow is fundamentally normal but that the spleen has exerted marked inhibitory effects.

It seems likely that the spleen, primarily a passive organ which removes spherocytes selectively, at times becomes unusually active, thus leading to both excessive

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This paper is dedicated to Dr. George R. Minor, whose memorable lectures on the physiopathology of diseases of the blood to our class at Harvard Medical School in 1921 did much to stimulate in me a sustained interest in hematology and in the mechanisms of blood formation and destruction. *William Dameshek*

phagocytosis and to hypersplenic inhibitory effects upon the marrow. The crisis may become so dangerous to life that emergency splenectomy may be required. Following splenectomy no further crises develop.

### METHODS

*Hemoglobin* For the most part, photoelectric colorimeter methods<sup>16</sup> were used, utilizing the Cenco and Evelyn instruments. Fifteen and six tenths grams per 100 cc. of blood were considered as the standard of 100 per cent normality.

*Serum bilirubin* The method of Malloy and Evelyn<sup>27</sup> was used with determination of prompt and indirect types. Values of less than 1.0 mg. of bilirubin per 100 cc. were considered normal.

*Fecal urobilinogen* The average daily urobilinogen output, in a four day stool collection, was determined by the method of Watson.<sup>49</sup>

*Reticulocyte and platelet counts* These were performed in the same preparations by the method of Dameshek.<sup>7</sup>

*Free erythrocyte protoporphyrin* This was determined by the method of Grinstein and Watson<sup>17</sup> after a period of observation and practice by one of us (M. B.) in the laboratory of Dr. Cecil J. Watson, University of Minnesota Medical School. The number of extractions performed was in direct relation to the amount of fluorescence seen.

*Plasma or serum iron determinations* The method of Kitzes, Elvehjem and Schuette<sup>44</sup> was used.

*Immunohematologic studies* Methods for the detection of circulating antibodies, including the use of bovine albumin as a diluent, were utilized as described by Neber and Dameshek.<sup>36</sup> Erythrocyte survival time studies were determined by a modified Ashby technic.

*Hypotonic fragility* The hypotonic fragility was determined in the earlier cases by the method of Daland and Worthley.<sup>5</sup> More recently, particularly in case B. S., the photoelectric method of Suess, Limentani, Dameshek and Dolloff<sup>44</sup> was used.

### REPORT OF CASES

In 1941, one of us<sup>6</sup> described three cases of hemolytic crisis which occurred within the same family during a ten day period. These two brothers and a cousin, all of whom lived in the same household, had in common a seventy year old grandfather who was known to have congenital hemolytic icterus. The latter's son, who was the father of the first two patients, also had the disease.

*Case 1* D. C. M., 2nd, aged 11, had been known to have chronic jaundice, anemia, and splenomegaly for several years. Mild recurring attacks of fever, weakness, pallor, and increased icterus had occurred about three times annually. On April 13, 1938, the patient suddenly developed pain and vomiting. On the following day jaundice and pallor were noted. Physical examination revealed an acutely ill boy, evidently in mild shock, who showed marked pallor and slight icterus of the sclerae and skin. The temperature was 103.5 F., the pulse rate 120-140 per minute. There was moderate splenomegaly, the spleen being felt 3-4 fingerbreadths below the left costal margin.

Examination of the blood showed hemoglobin, 36 per cent (Sahli), R. B. C., 1.25 M., W. B. C. 3800 per cu. mm. The red cells showed extreme spherocytosis and the average red cell diameter was 5.63 microns. On April 16 the red cell count had dropped to 1.01 M. and an emergency splenectomy was decided upon. The patient was then given large (probably excessive) amounts of intravenous fluids and blood both before and during splenectomy. Immediately following operation, he developed pulmonary edema and expired.

*Case 2* R. M. Jr. was a 12 year old boy and the brother of the first patient. Like his brother, he had been known to have had congenital hemolytic icterus for several years. He too had had several episodes (in the spring as a rule) of marked pallor and icterus associated with fever and malaise. On April 21, 1938, he developed fever, malaise, headache, and left sided abdominal pain. On the following day he was dizzy and weak. Physical examination revealed a temperature of 103.6 F., pulse rate 120/minute, the general appearance of acute illness, extreme pallor, slight jaundice, splenomegaly, and grave anemia. The hemoglobin was 30 per cent (Sahli) and the red cell count 1.22 M. The white cell count was 3000 per cu. mm. Extreme spherocytosis was seen and the hypotonic fragility showed beginning hemolysis at 0.64 per

cent NaCl, hemolysis being complete at 0.34 per cent NaCl. At the height of the crisis, it was noted that the reticulocytes numbered only 0.4 per cent. Three transfusions at four hourly intervals were given on April 23 with a resultant rise in hemoglobin to 70 per cent and in erythrocyte count to 3.3 M. Although clinical improvement was noted on the following morning, fifteen hours later, the red cell count had dropped to 2.56 M. Splenectomy was then performed. The next day, sharp increases in both the hemoglobin and erythrocyte levels were noted. Upon discharge from the hospital on May 6 the hemoglobin was 98 per cent and the red cell count 4.46 M. The subsequent course has been uneventful (see table 1).

*Case 3.* Marjorie McN., aged 5, was the cousin of the first two patients and lived in the same household. Like the others she had been known to have congenital hemolytic jaundice and had had several episodes of apparent hemolytic crisis. On April 22, 1938, she suddenly developed malaise and fever and complained of lower abdominal pain and headache.

TABLE 1—Hematologic Data, Case 2 (R. M. Jr.)

Date	R B C	Hgb	W B C	Plate lets	Hypotonic fragility	Serum bilirubin	Reticu- lo- cytes	Remarks
	<i>mil lions/ cu mm</i>	<i>%</i>	<i>thou sands/ cu mm</i>	<i>mil lions/ cu mm</i>	<i>% NaCl</i>	<i>mg %</i>	<i>%</i>	
4/22/38	1.22	30	3000		0.64-0.34	5.1	0.4	
4/23/38								500 cc transfusion
1:00 a m								400 cc transfusion
4:00 a m								
8:00 a m	2.54	47	3600					400 cc transfusion
10:00 a m								
12:00 p m	3.66	69					0.6	
3:00 p m	3.33	70						
4/24/38								
9:00 a m	2.56	68						Splenectomy
11:00 a m								500 cc transfusion
1:00 p m								
4/25/38	5.47	93						
4/26/38	4.67	89						
5/6/38	4.64	98					1.3	
5/17/38	3.72	80	8300	538			1.6	
6/14/38	4.61	89		1200			1.2	
10/18/38	5.06	102	7300	1113	0.52-0.26		0.2	
2/20/39	4.83	95	8400	740	0.60-0.04		1.4	
4/17/40	4.78	93	8900	1061	0.56-0.42	0.9		

Examination revealed an acutely ill little girl, with marked pallor and slight icterus. The temperature was 103 F and the pulse rate 120 per minute. The splenic edge was palpable one fingerbreadth below the left costal margin.

The hemoglobin was 35 per cent (Sahli). The red cell count was 1.7 M and the white cell count 5000 per cu mm. There was marked spherocytosis and the hypotonic fragility test showed beginning hemolysis at 0.80 per cent with complete hemolysis at 0.30 per cent. On the following morning, still at the height of the crisis, only 0.7 per cent reticulocytes were found.

Two transfusions, of 300 cc each, were given on April 26 and 27, following which splenectomy was performed. The spleen weighed 350 grams and showed extreme congestion of the pulp. Convalescence was uneventful. On May 4, the red blood cell count was 4.57 M, the hemoglobin 93 per cent and the white cell count 10,200 per cu mm. The subsequent course was characterized by a definite increase in the mean red blood cell diameter and a diminution of spherocytosis, although an abnormal hypotonic fragility persisted. The subsequent course has been uneventful (see table 2).



*Case 4* Mary D S, a 37 year old housewife, was admitted to the J H Pratt Diagnostic Hospital on April 14, 1944, complaining of marked weakness. Pallor had been noted since December, 1943, and the patient's husband had remarked upon the presence of scleral icterus for about a year. She had been asymptomatic, however, until April 8, 1944. At that time she developed malaise, generalized arthralgia and a shaking chill.

Examination revealed moderate fever, jaundice and slight stupor. The pallor became increasingly prominent and was associated with increasing weakness. On admission to the hospital there was barely perceptible icterus, a few petechiae in the region of the uvula and soft palate, and slight lymphadenopathy in the axillae. The heart showed a forceful apical impulse, and appeared somewhat enlarged to left. A grade II systolic murmur was heard at the apex and was transmitted to the base of the heart. The liver edge was tender and palpable 3-4 fingerbreadths below the right costal margin. The splenic edge was felt 3-4 fingerbreadths below the left costal margin. The blood showed hemoglobin, 5.3 Gm (34 per cent), R B C 1.53 M, W B C 6600, reticulocytes 0.3 per cent.

TABLE 2.—*Hematologic Data, Case 3 (M McN)*

Date	R B C	Hgb	W B C	Platelets	Hypotonic fragility	Serum bilirubin	Reticulo-cytes	Remarks
	<i>mil lions/ cu mm</i>	<i>%</i>	<i>thous sands/ cu mm</i>	<i>mil lions/ cu mm</i>	<i>% NaCl</i>	<i>mg %</i>	<i>%</i>	
4/24/38	1.70	35	5000		0.80-0.56			
4/25/38								
a m	1.98	35	5600					
p m	1.83	31	5000					
4/26/38	1.61	30			0.62-0.50		0.8	300 cc transfusion
4/27/38	2.25	38						Splenectomy + 300 cc transfusion
4/28/38	4.11	65	2100				0.8	
4/29/38	4.04	69	17000				0.3	
5/ 2/38	4.60	78					0.3	
5/ 4/38	4.57	93					0.4	
5/17/38	3.96	71					0.2	
6/21/38	4.12	80	13600		0.60-0.16			
7/12/38	4.27	78						
9/13/38	4.57	84	9500	1.540			2.3	
2/20/39	4.42	90		1.580	0.68-0.16			
4/17/40	4.46	89	7400	1.471	0.64-0.22		3.7	

Transfusions totaling 900 cc were given without reaction and the fever gradually subsided by the sixth hospital day. The reticulocytes rose to 5.4 per cent on the fifth hospital day. No abnormal circulating antibodies were found in the serum (salt solution used as the diluent).

Splenectomy was performed on April 21, 1944. This was followed by an uneventful recovery. The patient was seen last on July 3, 1947, at which time she was in excellent health. Blood studies were completely normal except for persistent spherocytosis (see table 3). Hemoglobin was 12.9 Gm (83 per cent), R B C 4.22 M, hematocrit 38 per cent, M C V 90, reticulocytes 0.8 per cent, platelets 1.73 M, and W B C 12,100 per cu mm. The serum bilirubin was 0.3 mg per cent (indirect).

*Case 5* H C, a 14 year old white boy noted vague malaise about March 10, 1946. He complained of severe headache and took a patent medicine containing acetanilid with some relief. On March 16 he suddenly developed sharp pain in the right lower abdominal quadrant, cramp-like in nature, and persisting for several hours. On March 17, the patient felt considerably improved but on March 19 an acute episode of vomiting occurred. On March 20 his physician noted fever of 104 F, a severe nonproductive cough and marked flushing of the skin. The patient was admitted to the hospital on that day.

On admission, he was acutely ill, and appeared weak, flushed and slightly cyanotic. He was apathetic and moderately pale. The oral mucous membranes appeared to be edematous and spongy. Scattered rales were heard at the base of the left lung. The heart rate was rapid but there was no evidence of enlargement and no bruits were heard. Marked tenderness was elicited in the lower quadrants of the abdomen, particularly on the right. The spleen seemed soft and was palpable four fingerbreadths below the left costal

TABLE 3 —Hematologic Data, Case 4 (M D S)

Date	R B C	Hgb	W B C	Platelets	Serum bilirubin	Reticulo cytes	Remarks
	millions/ cu mm	Gm	thousands/ cu mm	millions/ cu mm	mg %	%	
4/14/44	1 53	5 3	6600	084	2 3	0 3	Splenoectomy
4/15/44	1 90					0 2	
4/17/44	2 06	7 6	6600	188		1 9	
4/18/44	2 31	8 2	8400	268		5 4	
4/20/44	2 95	8 3	4200				
4/21/44							
4/22/44	3 75	10 5	23300				
4/25/44	3 35	10 4	15000				
4/27/44	3 36	10 6	13200				
5/18/44	4 32		16400				
4/13/45	5 03	14 4	14500				
6/20/46	4 35	14 6	16700	705	0 8	0 5	
7/ 3/47	4 22	12 9	12100	1 732	0 3	0 8	

TABLE 4 —Hematologic Data, Case 5 (H C)

Date	R B C	Hgb	W B C	Icterus index	Reticulo-cytes	Remarks
	millions/ cu mm	Gm	thousands/ cu mm		%	
3/20/46	3 10	8 9	9250	30	0 1	Splenoectomy
3/21/46	2 40	6 0	3850	20		
3/22/46	2 8	7 7	5000			
3/23/46						
3/25/46	3 8	10 6	22000			
3/26/46	3 9	9 7	30000			
3/27/46	4 7	10 7	40000			
3/28/46	4 2	10 9	35500			
3/29/46	4 0	11 6	20950			
3/30/46	4 3	10 9	24000			
4/ 1/46	4 4	11 6	17800			
4/ 3/46	4 4	11 9	17300			
4/ 5/46	4 3	11 8	13800			

margin. It was extremely tender and there was some spasm of the overlying abdominal muscles. A questionable bilateral Kernig reflex was elicited, there was a suggestion of a bilateral Babinski reflex and meningismus was present. Blood studies showed hemoglobin, 8.9 Gm, R B C 3.10 M, W B C 9250. The question of meningitis was considered seriously on the first day of admission. It was found that the patient had a rapidly progressive anemia. Penicillin therapy was not attended by improvement. On March 21, generalized lymphadenopathy was found. The nodes were bean sized and nontender. A few bleeding areas were noted in the oral mucous membranes, petechiae were present over the anterior chest,

and a generalized morbilliform eruption had appeared. The spleen was palpable four to six fingerbreadths below the left costal margin. Icterus was not noted. The blood examination now showed pancytopenia, i.e. anemia, leukopenia, thrombocytopenia and reticulocytopenia together with an extreme degree of spherocytosis. Actual blood findings were hemoglobin 6.0 Gm, R B C 2.40 M, W B C 3,850, reticulocytes 0.1 per cent.

On the basis of the above findings, the diagnosis of congenital hemolytic anemia in crisis was made. Splenectomy was performed after four transfusions had been given (two on the day of admission, one on

TABLE 5.—*Hematologic Data, Case 6 (P O N)*

Date	R B C	Hgb	W B C	Plate lets	Hypotonic fragility	Serum bili rubin	Fecal urobili nogen	Retic ulo cytes	Remarks
	<i>mil lions/ cu mm</i>	<i>Gm</i>	<i>thou sands/ cu mm</i>	<i>mil lions/ cu mm</i>	<i>% NaCl</i>	<i>mg %</i>	<i>mg / day</i>	<i>%</i>	
10/30/45	2.61	9.1	9000	0.213	0.52-0.34	1.8			
11/10/45	2.72	9.1	8250		0.56-0.30	2.0			
11/23/45					0.56-0.40			6.4	
11/28/45	2.88	10.4		493	0.52-0.38			10.2	
12/5/45								9.3	
12/21/45	2.82	11.0	9450		0.60-0.24			9.2	
1/9/46	2.91	11.0						9.1	
4/8/46	3.20	10.3	9000	820	0.56-0.36	1.4		7.7	
5/4/46						1.1			
5/20/46	3.45	12.0	4550		0.60-0.36	1.4			
5/27/46	3.89	11.0	6900				120		
6/3/46	3.76	12.3	5100	330	0.60-0.34	2.0		6.9	Beginning of minor crisis
6/5/46	3.24	12.3	3300			1.7		7.6	
7/7/46	3.59	12.7	5350		0.46-0.36	3.3		7.6	
1/10/46	3.21	10.7	6200		0.68-0.36	1.4		8.4	
6/21/46	3.48	11.7	5350			2.0	1050	7.8	
6/27/46	3.33	11.0	4650		0.46-0.34	1.5		8.1	
7/3/46	3.6	12.5	5000					7.8	
7/10/46									Splenectomy + 1000 cc blood
7/11/46	5.45	15.1	16900						
7/12/46	4.9	15.1	14000		0.64-0.36	1.2			
7/16/46	4.4	14.1	8200		0.68-0.36				
8/5/46	4.1	12.5	7200	500				0.8	
10/21/46	4.3	12.5	6750	443				0.6	
6/4/47	4.5	13.3	6550	907		1.0		1.2	

the third day and another just prior to operation) The spleen was greatly enlarged and at least six accessory spleens were present. The course after splenectomy was uneventful; this was particularly striking in view of the very critical condition of the patient before operation.

*Case 6 P O N* This 26-year-old woman was first seen in October, 1945. The patient came from a known family of familial hemolytic jaundice and represented the fourth generation in which the disease had been known.<sup>45</sup>

Intermittent jaundice had been noted first when she was 15. However, she seemed to be in good health until 1943, a few months after the death of her father of severe hemolytic crisis at the age of 55. At that time she was two months pregnant. She developed chills, fever and became very pale. The question of malaria was considered but malarial parasites were not found. The pregnancy proceeded uneventfully.

although a persistent yellowish tint of the sclerae continued. She complained of fatigue, recurring dizziness and some dyspnea on exertion.

Examination at this time during the sixth month of her second pregnancy elicited slight scleral icterus, moderate splenomegaly, a systolic murmur over the precordium, minimal pitting edema of the ankles and pretibial regions, and some superficial varicosities of the lower extremities. Examination of the blood showed R B C 3 10 M, hemoglobin 65 per cent, W B C 12,500 and marked spherocytosis with increased polychromatophilia and reticulocytes. The pregnancy was uncomplicated by any untoward incidents and a normal child was delivered on January 23, 1946. There was however continued fatigue and intermittent exacerbations in the chronic icterus. Slight pallor, icterus and splenomegaly continued to be the outstanding physical findings. Representative serial laboratory findings are recorded in table 5.

*Case 7.* The present study was initiated as the result of observations in this case and most of the illustrative figures are based on the data obtained.

Beverly S., a 13 year old white female of Portuguese origin, one of nonidentical twins, entered the J. H. Pratt Diagnostic Hospital on February 21, 1947. She complained of fainting spells, weakness, chills and fever of three days duration. About a week prior to admission she was stated to have contracted a mild upper respiratory infection. Four days before admission, her mother noted pallor. On the following day, the patient complained of dizziness, marked weakness and severe headache. The temperature rose to 104 F that evening. She vomited greenish bitter material and had severe vertigo. Nausea and vomiting persisted, and on the day of admission she was dizzy and very weak.

About one month before this admission there had been an episode of right lower quadrant pain unassociated with nausea or vomiting. Although appendicitis was suspected surgery was not performed. A week before admission another similar episode of abdominal pain occurred shortly after the onset of the last menstrual period. This subsided quickly. Several days before admission, the local physician remarked upon the fact that the patient was jaundiced. Neither dark urine nor light stools had been seen.

It was not possible to elicit any history of familial jaundice or anemia, either in the parents, grandparents, close relatives or in the five siblings.\*

The patient had always been noted to be considerably paler than her twin brother. She was a high school student and gave no history of exposure to chemicals or drug ingestion. Fava beans had been eaten occasionally by the family but not for about six months. The past history was irrelevant.

Examination on admission revealed an exceedingly well developed and well nourished girl who appeared very apathetic and critically ill, bordering upon shock. She was drowsy and hardly able to respond to simple questions. There was marked pallor and slight scleral icterus. The temperature was 100 F, pulse 120/minute, and blood pressure 105/50. The examination of the chest was negative. The spleen was readily palpable two to three fingerbreadths below the costal margin. The liver was not enlarged and there was no tenderness in the right upper abdominal quadrant. Representative blood findings are shown in table 6. In brief, at the time of admission they showed hemoglobin 4.0 Gm, (<25 per cent), R B C 1.3 M/cu mm, reticulocytes 0.0 per cent (1), W B C 15,500 per cu mm.

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\* Inasmuch as no evidence could be adduced in this case for the diagnosis of familial or hereditary spherocytosis, the reasons prompting our diagnosis of congenital spherocytosis deserve mention.

Detailed study of the parents, siblings and relatives was difficult because the patient came from a small town (Provincetown) situated more than 100 miles from Boston.

Through the cooperation of the patient's family physician, Dr. Thomas Perry, of Provincetown, it was found that her father, twin brother and the other two siblings showed no abnormal physical findings. Blood smears which were forwarded to us for examination were within normal limits. The mother was examined carefully but no significant physical or hematologic findings were elicited.

That the disease may be congenital and not familial has been noted, among others, by Wolman<sup>44</sup> and Race.<sup>40</sup> The latter observer found one affected child in each of three families although neither the parents nor the siblings nor the close relatives exhibited any evidence of the disease.

Lack of immune iso-antibodies in the blood serum, excellent clinical response to splenectomy, the persistence of spherocytosis, the normal survival time of transfused red cells before splenectomy and the complete lack of any evidence indicating an acquired hemolytic process of well defined etiology tended to support the initial clinical impression of an hemolytic crisis occurring in the course of congenital hemolytic jaundice even though evidence of familial disease was entirely lacking.

Jaundice was not a prominent feature during the hospital course. Very slight scleral icterus was noted during the first two hospital days after which it disappeared. Transfusions of 500 cc, 400 cc, and 500 cc of freshly drawn and citrated blood were administered on the first, second, and third hospital days. On the second hospital day there was slight improvement and following the third transfusion, the patient felt considerably better. At the outset, microspherocytosis dominated the blood smear. The reticulocytes were conspicuous by their complete absence during the first four hospital days despite the severity of the anemia and the hemolytic process. On the fifth hospital day, 0.1 per cent reticulocytes were found and then a gradual rise took place to a peak value of 12.6 per cent on the twelfth hospital day. At no time during the fourteen days of hospitalization did the red blood cell count or hemoglobin concentration approach normal values. Nevertheless, when she was discharged on March 7, the patient stated that she felt as well as she ever had. At that point, the following blood counts were present: R B C 2.76 M, hemoglobin 8.3 Gm (53 per cent), reticulocytes 10.4 per cent.

The patient was followed at frequent intervals and was then readmitted for splenectomy, which was performed on April 16, 1947, by Dr. C. Stuart Welch. Just before the operation the blood counts were as follows: R B C 2.61 M, hemoglobin 8.7 Gm (56 per cent), reticulocytes 10.4 per cent. The postoperative course was uneventful.

On June 23, 1947, the patient was asymptomatic and appeared to be in excellent general health. At this time the blood counts were normal (R B C 4.66 M, hemoglobin 14.6 Gm). When seen on September 5, 1947, the patient was in excellent health and had gained considerable weight. Her mother stated that she had never been so well (see tables 6-10, figures 1-8).

## ANALYSIS OF DATA

### *The Blood Picture*

In the report<sup>6</sup> from this laboratory in 1941, the following comment was made: The reduction in leukocytes, thrombocytes and reticulocytes in the cases reported here appears at first glance somewhat unusual since in the presence of an acute hemolytic process one would expect to find the evidences of increased regenerative activity on the part of the bone marrow, that is, leukocytosis, thrombocytosis and reticulocytosis. As further observations augment experience with the hemolytic crisis, in our own cases as well as in those reported in the literature, it becomes apparent that pancytopenia and reticulocytopenia are usually present.

In case 7, no reticulocytes (figure 5a, table 6) whatever were found at the height of the crisis. In fact, it was not until the fifth hospital day that the finding of 0.1 per cent reticulocytes was recorded. A gradual reticulocytosis then occurred, reaching an initial peak of 12.6 per cent on the thirteenth day of observation. Thereafter reticulocytosis was sustained until splenectomy was performed on the fifty-fifth day of observation. At the height of the crisis, there was also a moderate thrombocytopenia and granulocytopenia (23 per cent segmented and 1 per cent band forms) although the total white cell count was 15,500 per cu mm. By the fifth hospital day when reticulocytes were finally seen the thrombocytopenia and granulocytopenia had disappeared. Thereafter no significant changes in the white cells or platelets were noted until the postsplenectomy period when the expected thrombocytosis and granulocytosis occurred. The platelets have continued at unusually high levels.

The opportunity for such detailed studies was not afforded in the other cases, most of whom were seen under less ideal conditions for investigation. In general, however, these patients pursued a course quite similar to that of the seventh case. Thus in case 1, anemia and leukopenia were present at the height of the crisis.

TABLE C. Report on the Blood Chemistry of Case - B S \*

Day of observation	Date	H. B. C.	Hct.	W. B. C.	Granulo-cyte count per mm.	Granulo-cyte band form	Date of	Reticulo-cyte	Remarks
		Mill. per cu. mm.	per cent	Thous. per cu. mm.	"	"	Mill. per cu. mm.	"	
1	2-1-47	13	40	15500	23	-	0 35	0 0	Transfusion
2	2-2-47	15	40	14000	30	3	0 34	0 0	Transfusion
3	2-3-47	11	49	17000	57	11	0 34	0 0	Transfusion
7	2-24-47	13	60	11100	55	10	0 45	0 0	
5	2-25-47	15	74	22000	57	10	0 50	0 1	
6	2-26-47	20	60	10800	47	-	0 50	3 6	
7	2-27-47	23	60	9800	50	0		4 4	
8	2-28-47	15	74	8400	35	7	0 50	4 -	
11	3 3 47	27	75	9600	50	4	0 50	7 9	
12	3 4 47	25	85	6800	59	3		10 1	
13	3 5 47	27	85	11300	57	12	0 53	10 6	
14	3 6 47	16	75	11300	70	1		11 2	
15	3 7 47	18	83	14100	68			10 4	
21	3 13 47	18	80	10100	60	3	0 48	16 3	
32	3 24 47	10	87	8700					
49	4 10 47	33	88	12100	57	8	0 48	14 6	
51	4 12 47	29	93	10200					
53	4 14 47	31	88	10600	68		0 65	17 2	
54	4 15 47	30	93	8100	64	5		22 6	
55	4 16 47	26	87	8100	65	4	0 76	22 1	
55	4 16 47	31	93	29100					Splenectomy
56	4 17 47	36	87	18800	85	4	2 1	27 6	
57	4 18 47	34	83	19400	75	8			
58	4 19 47	35		17200	75	7	1 8	25 0	
59	4 20 47	34		14100	54	8	1 8	25 0	
60	4 21 47	35	87	9100	67	4	2 3	29 3	
61	4 22 47	34	93	8100	55	4	1 8	23 8	
62	4 23 47	33	95	8500	55	6	2 3	24 2	
63	4 24 47	33	95	8100	71	2			
64	4 25 47	37	104	6200	41	3	2 3	11 2	
65	4 26 47	38	107	12100	59	4	2 6	2 9	
67	4 28 47	37	110	10100	63	2	2 7	3 8	
68	4 29 47	38	110	9100	62	1	2 7	3 9	
69	4 30 47	36	107	7700	48		2 5	4 6	
74	5 5 47	36	110	6800	45	3	1 9	3 0	
76	5 7 47	37	110	9600	59	2	1 8	3 1	
78	5 9 47	37	107	7900	52		1 9	2 8	
100	5 31 47	44	121	11700	56	5	1 5	0 5	
123	6 23 47	47	146	8100	57		2 3	2 1	
197	9 5 47	44	133	8200	51	6	1 1	1 2	

\* The first day of observation coincided with the height of the hemolytic crisis. Transfusions of 500 cc, 400 cc, and 500 cc of freshly drawn and citrated blood were administered on the first, second, and third hospital days. Splenectomy was performed on the fifty-fifth day of observation.

In case 2, anemia, leukopenia and reticulocytopenia (0.4 per cent) were present. The postoperative period was characterized by a great outpouring of red blood

cells and platelets, unfortunately, reticulocyte and other blood cell counts were not available for adequate comparison. In case 3, reticulocytopenia (0.7 per cent), anemia, and leukopenia were present during the crisis. Following splenectomy the red blood cell count rose precipitously and leukocytosis occurred but no parallel reticulocytosis was seen. In case 4, reticulocytopenia (0.3 per cent) was present during the crisis, in addition there was marked anemia, thrombocytopenia and a

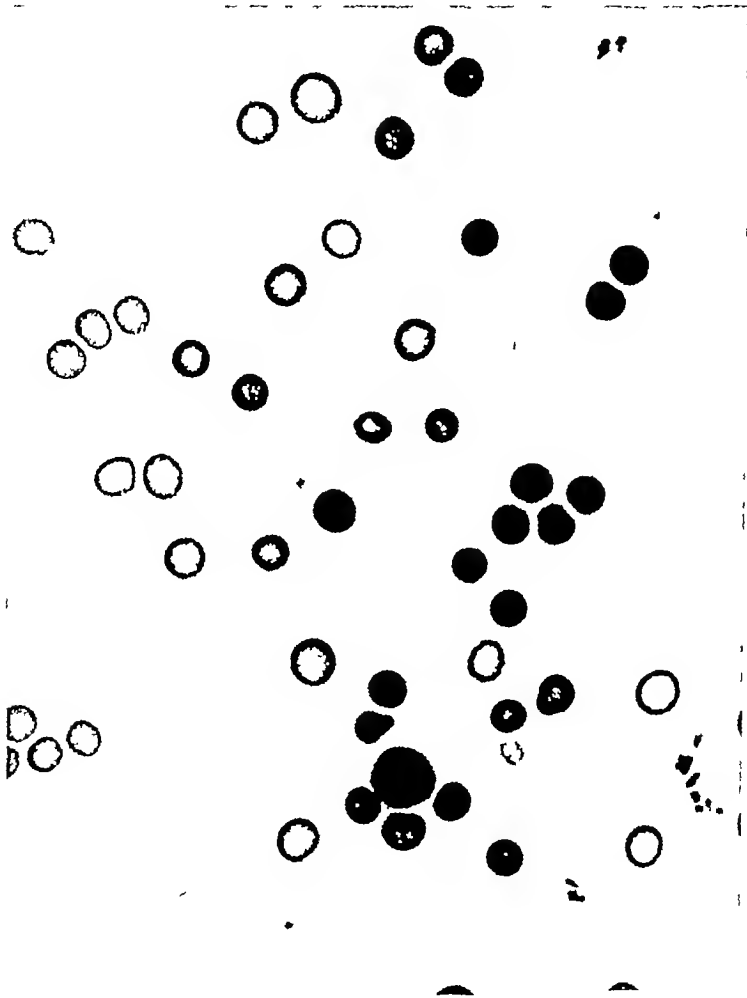


FIG. 1. CASE 7 (B. S.) Peripheral blood at the height of crisis, showing the predominance of micro-spherocytes.

normal or somewhat leukopenic white cell count of 6,600. By the fourth day a slight reticulocytosis of 5.4 per cent was present and thrombocytosis was developing.

Splenectomy was followed by a sharp rise in erythrocyte and leukocyte counts, but the data regarding reticulocytes and platelets are inadequate. In case 5, anemia, leukopenia, and reticulocytopenia (0.1 per cent) were present during crisis, and the blood platelets were definitely reduced on smears. In case 6, during a minor hemolytic crisis, the reticulocytes were increased to about 7 per cent but there was a definite leukopenia of between 3300 to 5300.

Extreme spherocytosis was an outstanding feature of the crisis in all our cases. There was very little evidence of the high stic type of blood cell picture seen when large polychromatophilic reticulocytes are also present. The picture is strikingly similar to that seen in the acute hemolytic anemia produced experimentally by large doses of hemolytic antiserum.<sup>4</sup>

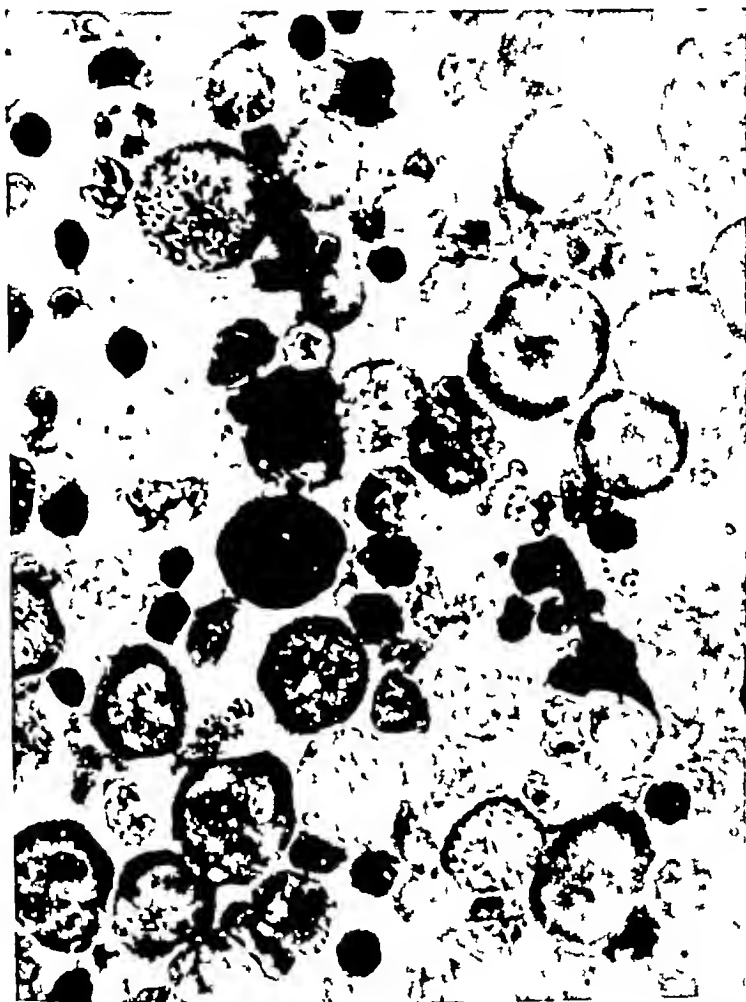


FIG. 2. CASE 7 (B. S.) Bone marrow at the height of crisis showing the accumulation of primitive erythroblasts (pronormoblasts, erythrogonocytes) and lack of maturation. The small mononuclear cells are lymphocytes.

In case 7, the Price-Jones curves (figure 6, table 8) of the red cells at the height of the crisis (and before transfusion) demonstrated that about 50 per cent of the red blood cells had a diameter of 4.8 micra or less, almost all the red cells had a diameter of less than 7.0 micra. Seven weeks later and just prior to splenectomy, 37 per cent of the red cells were 6.4 micra in diameter, and 25 per cent of the cells had a diameter of 7.2 to 8.8 micra. Five days after splenectomy, and probably due to an additional release of reticulocytes into the peripheral blood, there was a definite secondary peak at 8.0 micra. This latter figure represented the diameter of 22 per cent of the cells, although over 20 per cent still had a diameter of 5.6 micra.



TABLE 7 —*Beverly S. Special Studies*

Date	Day of observation	Fecal urobilinogen	Hypotonic fragility	Serum bilirubin
		<i>mg/day</i>		<i>mg %</i>
2/22/47	2		o 70-o 40	
2/24/47	4			1 1
2/26/47	6			1 2
3/ 5/47	13	255		
3/ 7/47	15			1 0
3/13/47	21		o 66-o 39	
3/24/47	32		o 69-o 42	
3/26/47	34		o 64-o 36	
4/10/47	49			
4/15/47	54			3 4
4/21/47	60			1 3
4/22/47	61		o 72-o 38	
4/25/47	64		o 72-o 40	
4/28/47	67			1 2
4/30/47	69	35		
5/ 5/47	74			o 8
5/ 9/47	-8			o 9
5/31/47	100	41		o 9
6/23/47	123			

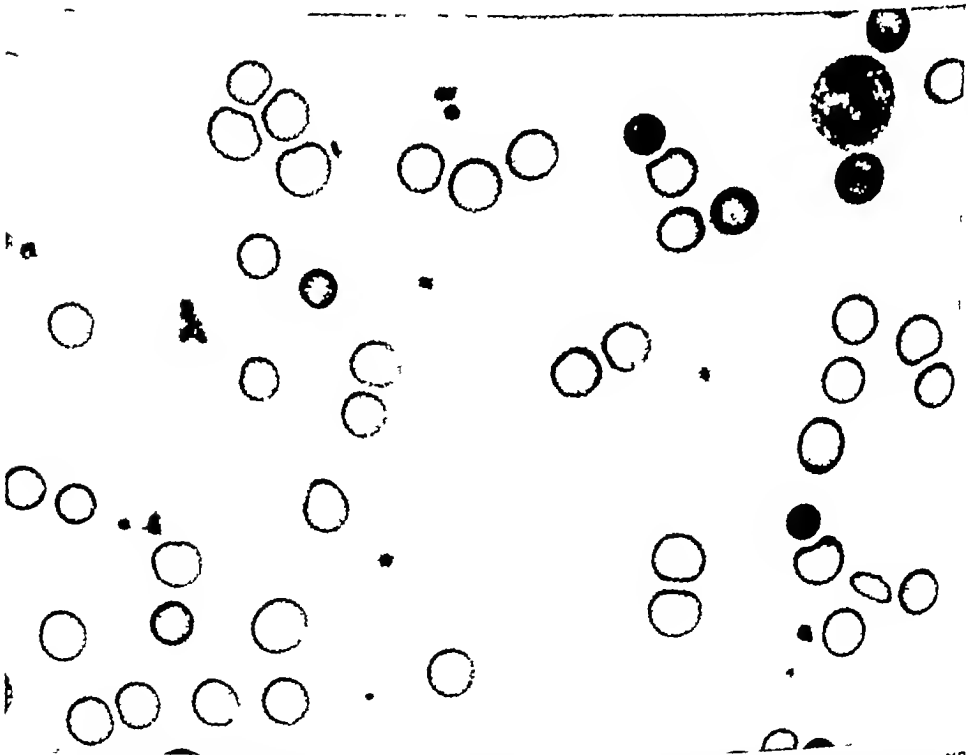


FIG 3 CASE 7 (B S) Peripheral blood five days following the height of the crisis Demonstrating the appearance of increased numbers of larger red blood cells and the relative or absolute decrease in microspherocytes

or less. Five months after splenectomy the Price-Jones' curve showed a main peak (39 per cent of cells) at 6.4 micra with a wide base due to the persistence of spherocytes.

That the small red cells are truly spherocytes is borne out, not only by their microcytic character and their abnormally dense appearance in the stained blood smear, but by their greatly decreased resistance to hypotonic solutions of sodium



FIG. 4. CASE 7 (B. S.) Bone marrow five days following the height of the crisis. Active progressive maturation of the normoblastic series is demonstrated.

chloride. All our cases of hemolytic crisis showed a greatly increased fragility. Occasionally, slight hemolysis has even been noted in concentrations of sodium chloride solution approaching that of normal saline. Extensive studies of hypotonic fragility were made only in case 7 (tables 1-4, 7, figure 7) in which the determinations were carried out by means of our newly devised photoelectric method.<sup>41</sup> By this method, curves of hemolytic increments, somewhat similar to Price-Jones curves, are obtained. These indicate graphically the different types of red cell population according to their thickness variation.

The blood picture immediately following splenectomy showed a dramatic increase in all the cellular elements. In fact the rapidity of increase of the red cells, white cells and platelets suggested a sudden outpouring of these cells from the marrow to the blood (figure 9)

### *Bone Marrow Picture*

Studies of the marrow by aspirations were performed in cases 2, 3, 4, 6, and 7. The relative time of marrow aspiration differed in these cases. As already mentioned, the conditions for study were ideal only in case 7. However in case 2, marrow was obtained at the height of the crisis and showed a maturation arrest type of erythropoiesis, closely similar to that seen in case 7.

Frequent aspirations of the sternal marrow both during and after crisis were carried out in case 7. At the height of the crisis, when no reticulocytes were found

TABLE 8—Representative Erythrocyte Diameters in Case 7 (B S)\*

Diameters in micra	2/21/47	4/14/47	4/21/47	9/5/47
	First smear at height of crisis before transfusion	Two days prior to splenectomy	Five days following splenectomy	Five months following splenectomy
	%	%	%	%
3 2	0 8	0	0	0
4 0	9 6	0	0	0 4
4 8	39 2	18 0	8 4	9 2
5 6	31 6	20 0	13 6	18 0
6 4	15 2	36 8	34 0	38 8
7 2	2 0	12 0	20 0	18 4
8 0	1 6	12 4	22 4	14 8
8 8		0 8	1 6	0 4

\* Marked microcytosis during crisis indicating extreme degree of spherocytosis and increase in size of red cells prior to splenectomy after termination of crisis

in the peripheral blood, the marrow preparations showed increased cellularity (figure 2). Granulocytopenia was active and the megakaryocytes were normal both in number and in platelet production. However, there was a striking abnormality of erythropoiesis, indicated by a complete lack of mature orthochromatic normoblasts (Normoblasts 'C') (figure 2). The great majority of the nucleated red blood cells were of the primitive variety i.e. erythrogonies or pronormoblasts. There were only small numbers of basophilic and polychromatophilic normoblasts (types 'A'' and 'B') (see table 9, figures 1 and 2).

From these observations, it was apparent in this case that there was a distinct maturation arrest of the erythropoietic tissue at or just beyond the erythrogonic (pronormoblast) level. On the fifth day, coincidentally with the appearance of a few reticulocytes, another sternal puncture was performed. This showed a normoblastic erythropoiesis with adequate numbers of polychromatophilic and orthochromatic normoblasts, indicating that the arrested maturation had run its course (table 9, figures 3 and 4). In four subsequent marrow aspirations, erythropoiesis continued to be hyperactive and no further evidence of maturation arrest was seen.

*Immunohematologic Findings and Erythrocyte Survival Time*

Studies of the serum for immune bodies were performed in cases 1 to 4 and in case 7. In the first four cases immune bodies were searched for using normal salt solution as a diluent, but in none of these cases were abnormal agglutinins or hemolysins discovered in the serum. In cases 6, (P O'N) and 7 (B S) iso-antibodies were discovered with the use of bovine albumin solution as a diluent, whereas negative results had been obtained using salt solutions. In case 6, (P O'N) an abnormal autohemolysin and isohemolysin, reacting best at 37 C, was discovered with the use of bovine albumin as a diluent, disappearing following the termination of the crisis. The survival time of transfused red cells was studied before and after splenectomy (Figure 8a). The curve of red cell disappearance before splenectomy was definitely abnormal and exponential in type, indicating that the abnormal antibody present was capable of destroying all types of red cells indiscriminately, including the patient's. Following splenectomy the survival time became normal again, coincidentally with the disappearance of abnormal antibody.

TABLE 9—*Bone Marrow Studies: Differential Count of Nucleated Red Blood Cells in Case 7 (B S)*

Date	Granulocyte erythroblast (G:E ratio)	Erythrocytes pronormoblasts	Basophilic normoblast (A')	Polychromato- philic normoblast (B')	Orthochromatic normoblast (C')
		%	%	%	%
2/22/47	3:1	79	20	1	0
2/27/47	1:2	3	8	48	41
3/5/47	1:1	7	11	55	27
4/17/47	1:5:1	7	19	62	12
4/25/47	1:5:1	4	8	40	48
9/5/47	1:5:1	4	26	59	11

The blood group of case 7 was B, Rh positive. Almost immediately following admission she was given a transfusion of 500 cc. of fresh group O Rh positive blood with added A and B group specific substances (Witebsky). On the second hospital day a transfusion of 400 cc. of fresh group O, Rh positive blood was given with added A and B (Witebsky) group specific substances. During the third hospital day the last transfusion, 1 c. of 500 cc. of fresh type B, Rh positive blood, was given. No reactions occurred with any of the transfusions. The survival time of the transfused Group O red blood cells was studied by the Ashby technic<sup>36</sup> and found to be normal, 1 c., 120 days (figure 8b). No transfusions were given in connection with the splenectomy and the disappearance of the transfused cells did not appear to be affected by the splenectomy (see figure 8b). Thus the two cases of hemolytic crisis differed radically with respect to red cell survival time, perhaps indicating somewhat different mechanisms.

*Bilirubin and Urobilinogen*

Jaundice was not an outstanding feature of these cases in crisis. The available data concerning serum bilirubin have been tabulated with the other blood studies in each case (see tables 1 and 7).

Determinations of the fecal urobilinogen were performed only in case 7. Due to various technical difficulties, this patient's stools were unfortunately discarded during the first eight days of her hospital stay. A four day stool collection was obtained during the succeeding period, when the hemoglobin varied between 7.8 and 8.5 grams per 100 cc. At this time the fecal urobilinogen excretion was 255

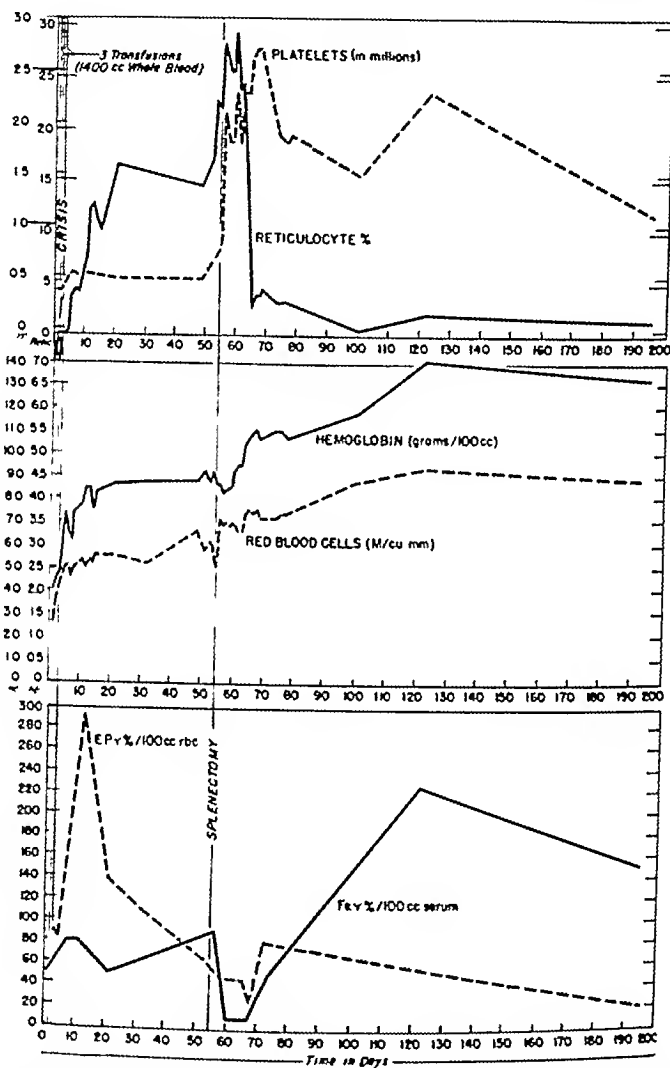


FIG 5a CASE 7(B S) Graphic representation of two hundred day period of observation, showing the variations in the significant serial studies. The lower graph describes the fluctuations in erythrocyte protoporphyrin and serum iron values.

milligrams per day. This is a definitely high value when related to the hemoglobin concentration, corresponding roughly with the excretion in a normal girl of about 500 mgs per day (normal 100-150 mgs per day). This indicated an approximately threefold increase in blood destruction at that time. Since the patient was improving at that time, it seems reasonable to assume that the fecal urobilinogen output during the first eight days may have been even greater. The two determinations of fecal urobilinogen obtained during the postsplenectomy period were within normal limits.

*Erythrocyte Protoporphyrin*

The normal range of concentration of free protoporphyrin in the intact circulating erythrocyte has been determined by Grinstein and Watson<sup>17</sup> to be between 15 and 40 gammas per 100 cc of red blood cells, the usual normal value is in the vicinity of 30 gammas per cent or lower

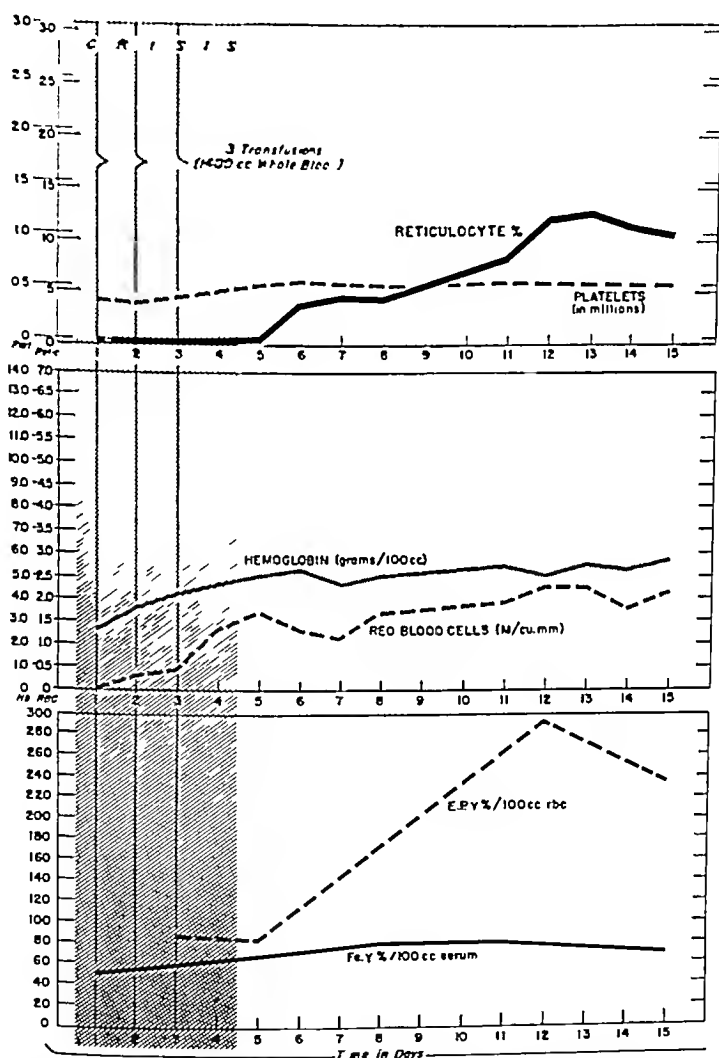


FIG 5b CASE 7 (B S) Close-up of certain values in the first fifteen days of the clinical course

Serial determinations of free erythrocyte protoporphyrin (EP) were obtained only in case 7 (table 10, figure 5). At the height of crisis, at a time when reticulocytes were completely lacking, the EP was 87.8 gammas per cent, well above normal limits. This was perhaps due to an increased erythropoietic activity just prior to the development of the crisis. As recovery from the crisis progressed and reticulocytes appeared in the blood (coincident with marked marrow erythropoiesis) the EP concentration rose rapidly, reaching peak levels of 292 gammas per 100 cc. On the twelfth day of observation, the EP gradually decreased until on the sixty-

eight day it reached a normal value of 25 gammas per cent. Following this, it was found that a state of hypoferremia had developed. This was mirrored, soon later, by a definite increase in the EP. Following the administration of ferrous sulfate, the EP fell again to within normal limits.

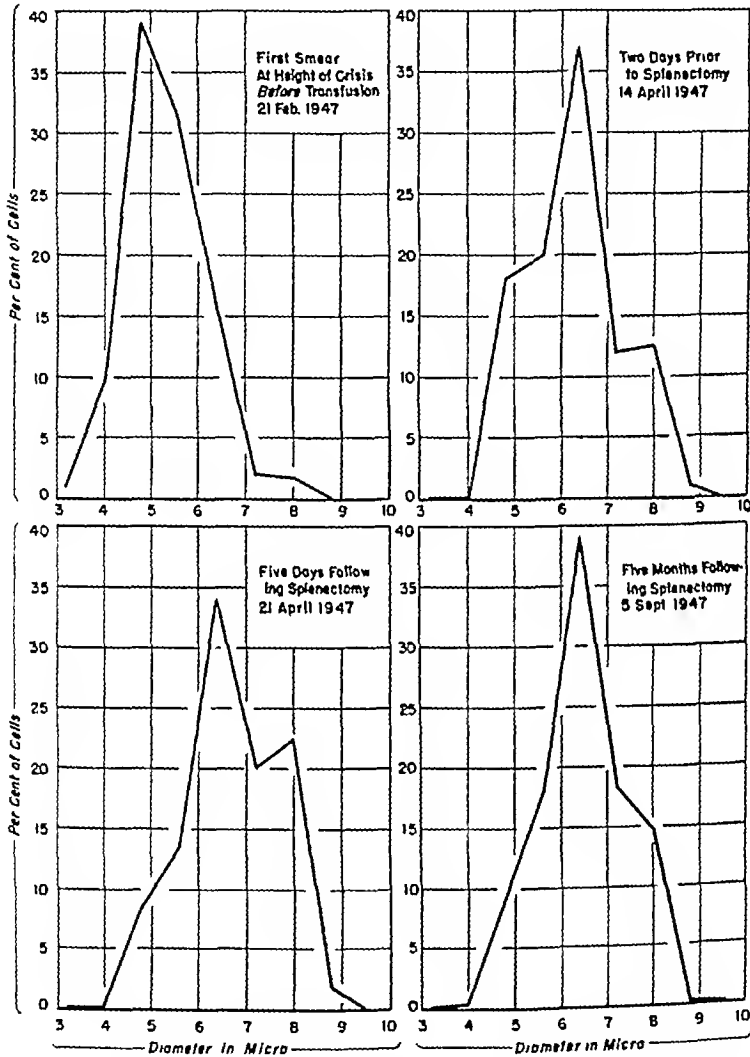


FIG. 6 CASE 7 (B S) Price-Jones curves of representative blood smears during the clinical course

### Serum Iron

The concentration of serum or plasma iron and its normal range has been defined by several investigators<sup>31 32 39 47 48 52</sup> as ranging between approximately 50-180 gammas per 100 cc serum or plasma.

In case 7, B S, a serum iron determination at the height of the crisis was 50 gammas per 100 cc concentration, i.e., at the lowest range of normality (see table 10). The patient was not given iron therapy at this time, her only sources of iron being through an unrestricted diet and three transfusions. After the transfusions had been given, a value of 80 gammas per cent was obtained. During the postcrisis

(presplenectomy) period, this comparative plateau of serum iron values seemed to have no correlation with the great fluctuations in free erythrocyte protoporphyrin

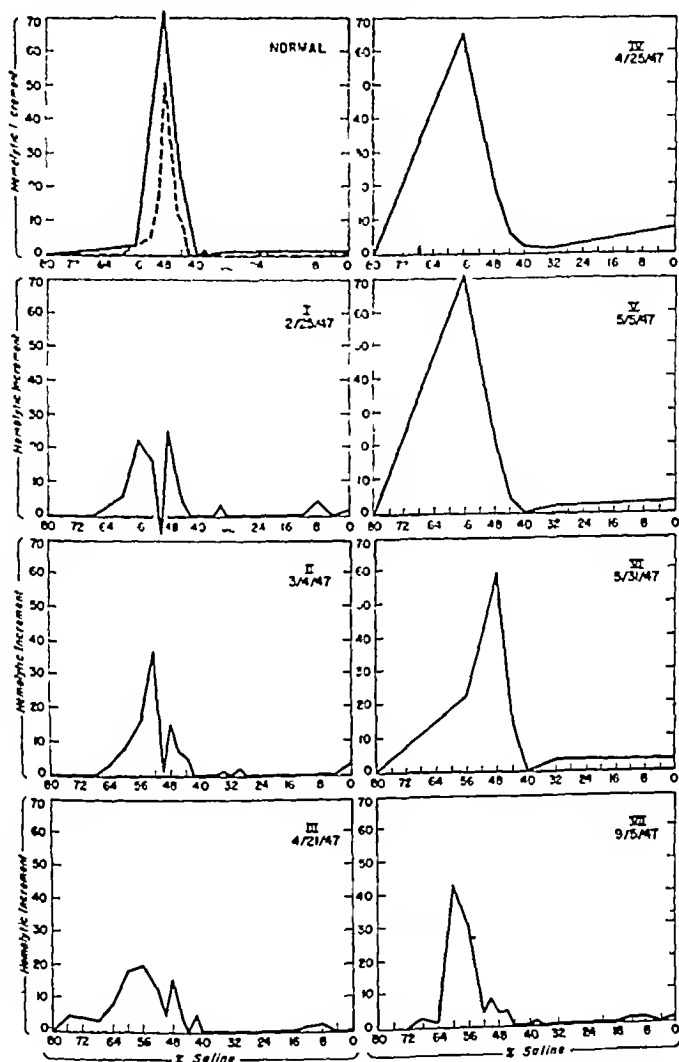


FIG 7 CASE 7 (B S) CURVES OF HYPOTONIC FRAGILITY

Two methods were used to determine the curves illustrated above. These differed only in detail. One involves the use of 29 tubes containing 29 serial saline dilutions from 0.80 to 0.0 per cent NaCl. In each of these the per cent hemolysis was determined, using the Evelyn photoelectric colorimeter. The other uses only 7 tubes, containing the dilutions which have been found to be most critical: 0.80, 0.56, 0.48, 0.44, 0.40, 0.32, and 0.0 per cent. The graph of the normal fragility shows curves from both of these methods, the solid line indicating 29 dilutions and the dotted line, 7 dilutions. Graphs I, II, III, and VII depict 29 tube studies, whereas IV, V, and VI show curves from the 7 tube test.

The hemolytic increment means simply the change in degree of hemolysis from tube to tube as hypotonicity increases (from above, downwards). Therefore, at each point of changing saline concentration, the per cent hemolysis is obtained by the Evelyn photometer, this is compared with the per cent hemolysis in the previous tube, and the per cent difference, or the hemolytic increment, is obtained.

However, two days following splenectomy, a value of 90 gammas per cent was obtained, that this was followed by a sharp drop of the serum iron value to 10 gammas per cent (marked hypoferrremia). At this point the EP values had reached



normal limits and it was predicted that, on the basis of the hypoferremia, the EP concentration would rise. This actually occurred (table 10, figure 5a).

Ferrous sulfate was then given, and a significant rise in serum iron occurred. It is possible that the drop of the serum iron value was due to the continued depletion of the depot iron stores by the persistent demands of hyperactive erythropoiesis coupled with menstrual iron loss.

## DISCUSSION

### *The Hemolytic Crises of Hereditary Spherocytosis*

Familial spherocytosis is characterized by a chronic hemolytic anemia of variable severity in which spherocytosis and increased hypotonic fragility are prominent features.

The chronic course of the disease is often punctuated by minor and major exacerbations in the intensity of the hemolytic process. Minor episodes are characterized by malaise, low grade fever, increased pallor and icterus. These episodes last a few days or a week, subside spontaneously and quickly, and in fact often go unrecognized.

The major exacerbations may be so severe as to endanger life. Beginning like an acute febrile illness, they progress rapidly with the development of abdominal discomfort, marked pallor, dizziness, nausea and vomiting, chills and fever. Diarrhea may occur and discharges of bile and grossly bile stained stools may be present. During the height of the crisis, the patient may go into shock or a shock-like state, stupor and syncope are common. Examination reveals an extremely sick-looking person, who is markedly pale but only slightly jaundiced. The pulse is rapid and feeble and the blood pressure is often low with a low pulse pressure. The spleen often becomes considerably enlarged, as compared with its previous size, and is frequently tender.

The management of the crisis has been described in a previous paper.<sup>6</sup> The use of fresh whole blood is of paramount importance. Transfusions not only add red cells but have a well-defined effect on the blood (and plasma) volume. The sudden lowering of the red cell count to levels of 3.5 M to 1.5 M may result in the symptoms of shock. Therefore, appropriately spaced transfusions may be life-saving. It should be noted, however, that transfusions probably have little if any effect on the mechanism of the crisis itself, and may well be overdone. (Overtransfusion was probably responsible in large part for the fatal determination in case 1.) By the judicious use of transfusions and intravenous fluids, an adequate preparation of the patient for splenectomy is possible. This type of management results in a quieter and less 'toxic' patient, and thus in a far better surgical risk and a smoother convalescence.

Another remarkable feature of the hemolytic crisis is its occurrence in rapid succession in several members of the same family. Scott<sup>12</sup> reported in 1935 the serial onset of acute blood crises in an entire family. In Dedichen's series,<sup>13</sup> reported in 1937, 13 members of 2 neighboring families living in a small town in Norway developed crises within a few days of each other. One of us<sup>6</sup> in 1941 reported three cases of familial crisis occurring within ten days. In 1945, Horne, Lederer,

Kirkpatrick and Leys<sup>21</sup> reported hemolytic crisis in the mother and four children of a family of eight, and also in the mother's cousin who lived nearby. In these cases, too, the illness developed in rapid succession in one individual after the other. A few days as a rule elapsed between the onset of the crisis in the various cases.

The occurrence of the crisis in several members of one family and, in two instances, in neighboring families, has naturally led to investigation for an extrinsic cause for the crisis. Thus, in Dedichen's large series,<sup>12</sup> it was suspected that some sort of highly contagious respiratory infection was responsible, at least for initiating the crisis. This appeared to be substantiated by the development of an acute febrile illness in one of the siblings who did not have the congenital hemolytic disease and during the period when the others were having hemolytic crises. Horne *et al.*<sup>21</sup> placed a ferret in the home of their first family to develop crises in the belief that a virus infection such as influenza might be the responsible agent. The ferret was returned to the laboratory and died shortly thereafter, with symptoms of a nasal discharge and conjunctivitis.

The occurrence of the crisis in families, with 'incubation periods' of a few days between cases, is strong presumptive evidence that an infection is responsible for at least initiating or precipitating the crisis. However, since no real proof of an infectious etiology has been adduced, it is also possible that no infection is present but that fever, rapid pulse, headache, malaise, nausea and vomiting are the results of rapid blood destruction alone.

A final feature of the hemolytic crisis is that it occurs only in the presence of a spleen, i.e., following splenectomy, crises either do not occur or are extremely unusual. This is another indication of the importance of the spleen in initiating crisis (cf. below).

Lowe<sup>26</sup> who studied a case during crisis, in which the red cells, hemoglobin, and reticulocytes continued to diminish despite therapy concluded that suppression of marrow function might be responsible. Unfortunately, studies of the marrow were not performed. In the cases of Horne *et al.*,<sup>21</sup> mentioned above, in which complete lack of reticulocytes was reported at the height of the crisis, a marrow examination was performed in only one case and then on the eleventh day of illness when the patient was improving. These authors referred, however, to a statement by Josephs,<sup>23</sup> who believed, on theoretical grounds, that a depression of marrow activity might be vital to the pathogenesis of the crisis.

Owren<sup>38</sup> in a recent comprehensive article reported crises in six cases of congenital hemolytic jaundice and also stressed the occurrence at the height of the crisis of leukopenia, thrombocytopenia, and reticulocytopenia. He was able to study one case before, during, and after crisis and obtained serial examinations of blood and marrow. Owren concluded that there was an erythropoietic 'aplasia' during crisis. He believed that the sudden drop in red cell count occurring in crisis could be adequately explained on the basis of an 'aplastic reaction on the part of the erythropoietic tissue and went so far as to deny that the factor of hemolysis played any role whatever in the crisis. He suggested that the term 'hemolytic' for the crisis be dropped.

Serial punctures of the marrow in case 7 indicated that the marrow reaction passes through several distinct stages which could be correlated with the reticulocyte picture. During the height of the crisis, when reticulocytes were lacking, the marrow (figure 2, table 9) showed a nucleated red cell population consisting almost entirely of the most primitive cells, i.e., erythrogones (pronormoblasts) and some basophilic normoblasts. Several days later and simultaneously with a slight increase of the reticulocytes in the blood, there was a complete reversal (figure 4, table 9) in the marrow picture. Most of the nucleated red cells were of the polychromatophilic ('B'') variety. Later, as the reticulocyte peak had passed and the

TABLE 10—*Serial Erythrocyte Protoporphyrin and Serum Iron Determinations in Case 7 (B S)*

Date	Day of observation	Erythrocyte protoporphyrin (EP)	Serum iron	Remarks
		<i>gammas per 100 cc packed R.B.C</i>	<i>gammas %</i>	
2/21/47	1		50	Period of crisis
2/23/47	2	87.8		
2/25/47	5	83.3		
2/28/47	8		80	Postcrisis
3/ 3/47	11		80	
3/ 4/47	12	292.0		Spleneectomy April 16
3/13/47	21	137.6	50	
3/24/47	32	109.2		
4/14/47	53	65.0		
4/17/47	56		90	
4/19/47	58	47.3		
4/21/47	60		10	
4/26/47	65	44.0		
4/28/47	67			
4/29/47	68	25.0		
5/ 1/47	70		30	
5/ 3/47	72	78.5		
5/ 5/47	74		50	
6/23/47	123		225	
9/ 5/47	197	30.0	150	

marrow picture was that as ordinarily seen in congenital hemolytic jaundice, most of the normoblasts (table 9) were of the mature variety. Thus the reticulocytopenia of the crisis could be explained adequately by a maturation arrest of the nucleated red cells of the marrow at the erythrogonic level, with a resultant lack in the production of mature non-nucleated erythrocytes.\*

\* The large primitive red cells seen in the crisis have been referred to by some observers<sup>17</sup> as megaloblasts. The concept of what a megaloblast is has been confused in the literature, in our laboratory this term is used for the nucleated red cell series as seen typically in pernicious anemia and related states. The nucleus of the megaloblastic cell retains a relatively primitive chromatin mesh (which recalls that of the reticulum cell) until just before pyknosis. The identity of the megaloblastic series has been discussed by Jones.<sup>22</sup> Our studies indicate that the preponderant nucleated red cell—seen in the bone marrow during crisis—is not a megaloblast but is a primitive red cell of the erythrogonic (pronormoblast) type. What

The maturation arrest of the nucleated red cells in the bone marrow is apparently not paralleled by an arrested maturation of the granulocytes, which indeed appear to be increased in number, both in an absolute as well as a relative fashion. Furthermore, the relative proportions of the different types of granulocytes in the marrow are within normal limits, suggesting that a maturation defect of these cells is lacking. These findings in the marrow, in association with the leukopenia and granulocytopenia of the peripheral blood, might indicate that a "block" phenomenon is present, i.e., although maturation of the granulocytes is proceeding in a normal fashion, the mechanism controlling their delivery from the bone marrow to the blood is perhaps defective.

Such a phenomenon occurs typically in various types of splenomegaly in which a possibly exaggerated activity of the spleen, i.e., hypersplenism,<sup>10</sup> is present.

TABLE II—Comparison of Blood Counts during Crises in the Seven Cases

Case	R B C	W B C	Reticulo cytes	Remarks
	millions/cu mm	thousands/cu mm	%	
I D C M Jr	1.25	3800	—	Minor hemolytic crisis
II R M Jr	1.22	3000	0.4	
III M McN	1.7	5000	0.7	
IV Mary D S	1.53	6600	0.3	
V H C	2.40	3850	0.1	
VI P O N	3.24	3300	7.6	
VII B S	1.3	15500 30% gran- ulocytes	0.0	

This is present in such diverse conditions as portal hypertension (cirrhosis of the liver), chronic infectious splenomegaly, including malaria, syphilis, tuberculosis, rheumatoid arthritis and Boeck's sarcoid, Gaucher's disease, various primary neoplasms of the spleen, and finally in many cases of idiopathic splenomegaly. Leukopenia, neutropenia and thrombocytopenia, i.e., pancytopenia, are present, although the bone marrow itself is hyperplastic. Occasional cases of "hypersplenic" hemolytic anemia show low reticulocyte counts with a well-defined maturation arrest of nucleated red cells at the erythrogonic level. In these cases, and from an analogy with other instances of hypersplenism,<sup>10</sup> we have come to the conclusion that the hemolytic crisis is based on a suddenly developing splenic disturbance. This, in turn, may be of infectious origin. No definite evidence of infection has been advanced as yet, but the occurrence of successive cases in the same family is strong presumptive evidence of this possibility. Doan<sup>13, 14</sup> contends that the splenic hemolytic mechanism may be readily unbalanced with the result that numerous minor crises may develop during an individual's lifespan. This might develop, for example, in the presence of certain acute infectious states.

little maturation occurs beyond this primitive stage is productive of a normoblastic and not of a megaloblastic type of erythropoiesis.

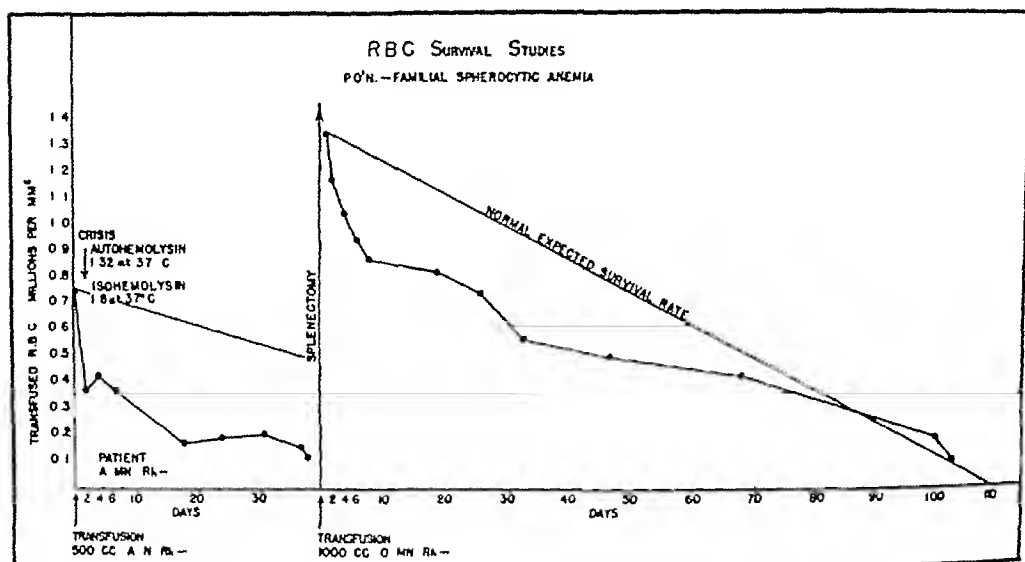


FIG 8a CASE 6 (P O N) Survival studies (Ashby technic) during crisis and after splenectomy The red cell survival time was diminished during crisis, and became normal after splenectomy

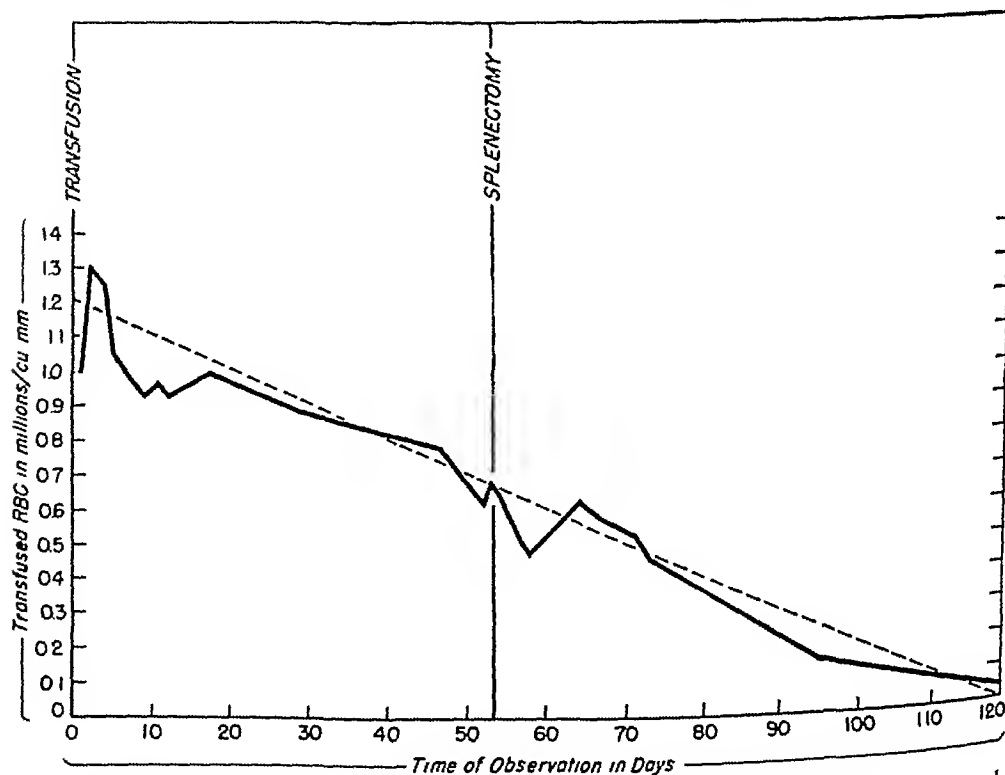


FIG 8b CASE 7 (B S) The rate of disappearance of the transfused red blood cells pursues a straight line course, i.e., normal survival time

It is possible that the postulated splenic abnormality leads (a) to an increase in hemolytic activity with resultant extreme spherocytosis and rapidly progressive anemia, and (b) to various inhibitory "hypersplenic" effects. Such effects upon the

bone marrow might result in, (a) a "block" phenomenon in which granulocytes are prevented from being delivered normally to the circulating blood, (b) a reduced production of platelets from megakaryocytes and, (c) in maturation arrest of red cells at the erythrogon (pronormoblast) level. These phenomena would lead to reticulocytopenia, leukopenia, granulocytopenia, and thrombocytopenia. Thus, the various features of the hemolytic crisis may be conceived of as being due to an extreme degree of hypersplenism, causing simultaneously both excessive

### COMPARATIVE BLOOD COUNTS BEFORE & AFTER SPLENECTOMY

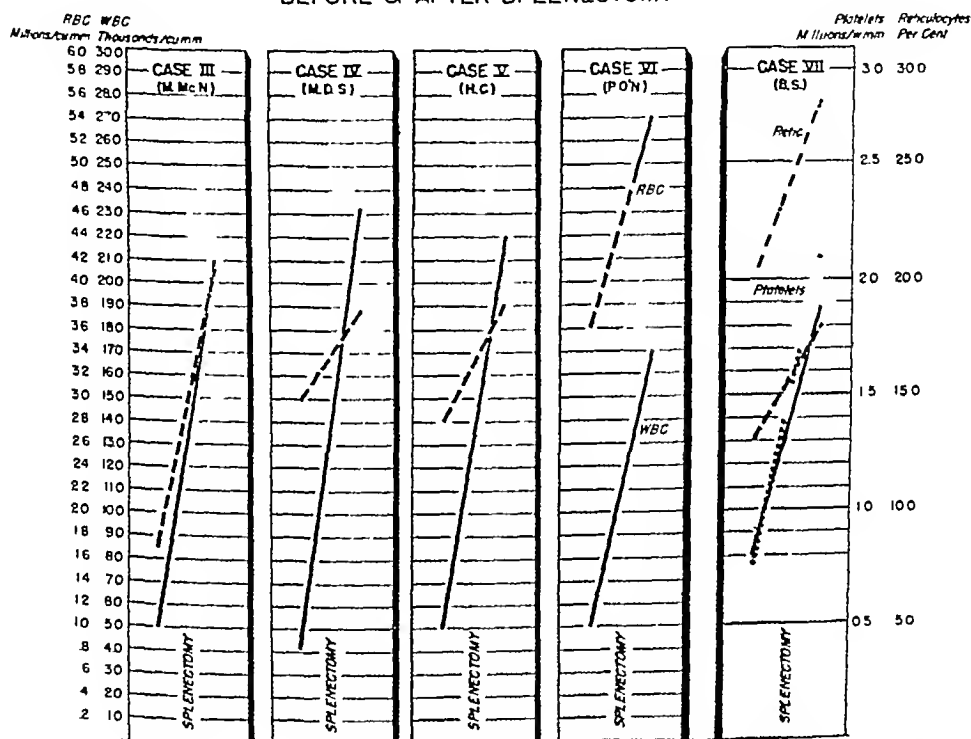


FIG. 9. Comparative blood counts before and after splenectomy in cases 3-6, 7. The darkened portions represent the period immediately preceding splenectomy. In the white columns are the values obtained immediately following splenectomy. Reticulocyte and platelet determinations are included only in case 7.

hemolysis and maturation arrest of the nucleated red cells. This would naturally lead to an extremely rapid development of anemia (figure 10).

The exact mechanisms by which blood becomes destroyed during the hemolytic crisis remain quite obscure. There is no evidence of intravascular hemolysis as indicated by hemoglobinemia and hemoglobinuria. The finding of abnormal iso-antibodies in two of our cases (autohemolysis in case 6, autoagglutinin in small concentration in case 7) suggests an immune body type of autohemolytic activity, as described in previous papers.

This is confirmed by an abnormal survival time of transfused red cells in our case 6. No evidence of erythrophagocytosis is evident in the studies of splenic

histology The chief histologic feature of the removed spleen is the great 'pooling' of blood within the pulp which shows large numbers of ghost cells and cells in various stages of hemolysis with the sinusoids appearing to be almost empty. It would appear that the blood has largely left the sinusoids for the pulp where it is 'trapped'. It is possible that an infection may so alter the capillary permeability of sinusoids that blood "leaks" from them into the pulp where various mechanisms

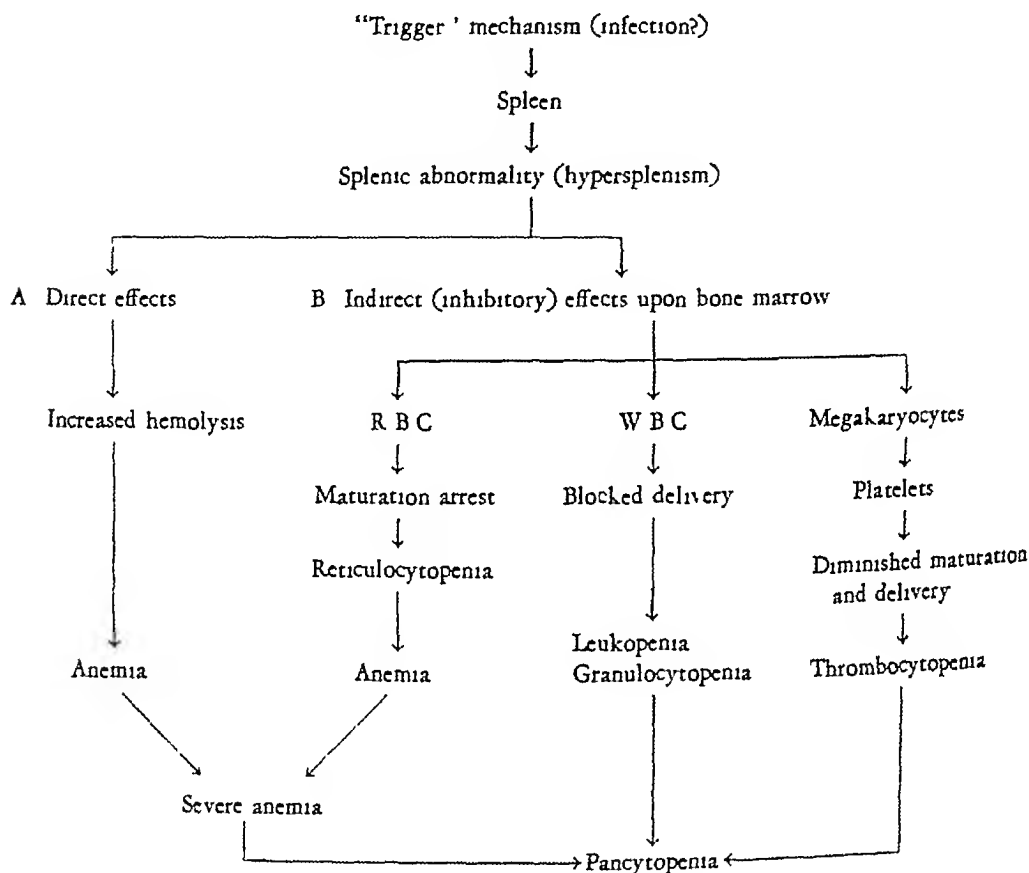


FIG 10 —THE HEMOLYTIC CRISIS

such as erythrostatics, mechanical trauma, and abnormal antibody activity might combine in the destruction of excessive numbers of red cells

We do not believe, as Owren<sup>28</sup> maintains, that the events in the hemolytic crisis can be explained on the basis of a suddenly developing marrow aplasia. In the first place, the marrow is by no means aplastic. Secondly, the marrow is evidently capable of an extremely rapid increase in activity when the spleen has been removed. This is borne out by the sudden rise in red cells, white cells, and platelets which occurs after splenectomy. This is due, we believe, to the removal of a highly deleterious organ, having inhibitory and phagocytic effects. Furthermore, Owren's concept does not explain the extreme degree of spherocytosis which regularly occurs during crisis and which indicates, according to our investigations,

a marked degree of hemolytic activity. Studies of the fecal urobilinogen should settle this problem. These were not carried out by Owren, nor in our own series, except in case 7, where it was distinctly elevated. Dr. Jonah Li (University of Oregon Medical School) recently studied a family of five individuals having hemolytic crises occurring within ten days of each other. All the cases showed blood pictures similar to those described above, with extremely low reticulocyte values. In two cases, fecal urobilinogen determinations at the height of the crises, showed values that averaged approximately 1500 mgs per day, i.e., about 20 times the normal value, taking into account the severe anemia. At the termination of the crises, the fecal urobilinogen values became very low.

A final indication of the direct relationship of the spleen to the crisis is the complete disappearance of all tendency to crisis following splenectomy. Whereas before operation the red cell count is subject to well-defined and even to marked fluctuations, this does not occur following operation. Spherocytosis continues to be present but the sudden extremes in this abnormality in association with a greatly increased anemia, do not develop. This is another indication that the spleen has an active influence in blood destruction and in initiating the various events of the hemolytic crisis.

#### *The Possible Relation of the Events in the Hemolytic Crisis to the Normal Hemolytic Activity*

The sudden development of extreme spherocytosis and extreme red cell destruction in the crisis suggests strongly an extrinsic activity, i.e., a factor outside the marrow resulting in changes affecting the non-nucleated red cell. The theory most commonly held for the spherocytosis of the familial disease is that it is the product of an abnormal type of erythropoiesis. Since spherocytosis is most marked in the crisis, one would expect to find some evidence of this abnormality in the bone marrow. However, this is by no means the case, either in the chronic form of the disease or during the crisis. In the chronic form, the marrow shows marked erythroblastic hyperplasia, maturation is orderly, and a gradual reduction in size of the nucleated red cells occurs as the mature orthochromatic stage is reached. No evidence for the development of spherocytosis can be seen in the first stages of the life span of the non-nucleated erythrocyte (i.e., the polychromatophilic red cell) in which the cells show a diameter greater than normal. The discrepancy between the relatively large polychromatophilic reticulocytes and the small orthochromatic spherocytes is quite striking, suggesting that some abnormal mechanism modifies the rather young circulating erythrocytes making them spherocytes. This is noted, even more strikingly, in acute acquired hemolytic anemia with abnormal iso-antibodies and can be reproduced experimentally.

In the extreme degrees of spherocytosis, as seen in the hemolytic crisis, one would expect, if the theory of a bone marrow abnormality were correct, to find an extreme degree of spherocytic development in the marrow. Instead the marrow shows very large primitive cells with an almost complete maturation arrest. We believe that this can only indicate that the spherocytes become spherocytic outside the marrow perhaps by some sort of abnormal intravascular mechanism. Since the crisis may



represent an exaggeration of the normal hemolytic mechanism and since spherocytosis is so marked in the crisis, we believe that the spherocyte does not necessarily indicate an inherent defect of erythrocyte formation, but rather the end result of an abnormal type of hemolytic mechanism

That abnormal iso-antibodies are not found customarily in familial spherocytosis, does not rule out their presence. These antibodies may be of such minor intensity or adsorbed in some fashion to the red cell surface, as to make their detection difficult by methods now available. The failure to find abnormal antibodies need not necessarily indicate that they are lacking since methods for their detection may still be imperfect. In the past two years alone, two new methods have been introduced: the use of bovine albumin as a diluting medium and the Coombs antiglobulin test. These tests have resulted in the frequent finding of hemolytic antibodies, undetected by methods previously in use. They suggest that, with the years, other methods, even more sensitive, may become available.

Similar reasoning holds true for the normal life span of red cells introduced into the circulation of an individual with the disease. The fact that the "foreign" red cells are not destroyed more rapidly than normal has led to the widespread conception that the disorder must therefore be due to an inherent defect of the individual's own red cells. This need not necessarily be true since the abnormal hemolysis may be autospesific (i.e., active only against the individual's own red cells). Furthermore, our finding in one case of crisis, of a greatly diminished red cell survival time (see figure 8a, case 6) might indicate that in crisis the hemolysin activity may become so outspoken that it will not only cause a rapid destruction of the individual's own cells but also result in an exponential type of destruction of introduced "foreign" cells.

#### SUMMARY

The course of hereditary spherocytosis (congenital or familial hemolytic anemia) is subject to major or minor exacerbations or crises. Pancytopenia, reticulocytopenia, and extreme spherocytosis characterize the major crises, during which hypersplenic effects appear to play a major role. These are characterized by the combination of (1) an unusual degree of hemolysis with (2) inhibitory effects upon maturation and delivery of bone marrow cells. At the height of the crisis, an extreme degree of maturation arrest in erythropoiesis is present. Splenectomy, which is often urgently necessary, results in a very rapid increase in all the cellular elements of the blood, confirming the phagocytic and inhibitory effects of the abnormal spleen. Following splenectomy, no further crises occur. The presence of successive cases in the same family suggest the possible role of infection as a precipitating or trigger agent in initiating an abnormal splenic mechanism leading to crisis. The cause of the hereditary spherocytosis is commented upon and evidence bearing upon an autospesific mechanism is discussed.

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# JAUNDICE AND THE SULFONAMIDE DRUGS

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IT IS NOW well established that jaundice is one of the toxic manifestations of sulfonamide chemotherapy. Although it is usually possible to recognize this complication, sulfonamide-induced jaundice may be difficult to differentiate from toxic hepatitis occurring as a complication of the infectious process itself as well as from sporadic instances of infectious hepatitis and serum jaundice. Since the sulfonamide drugs are widely used in medical practice, it is perhaps of some interest to review the distinguishing characteristics of toxic hepatitis caused by these compounds. The establishment of a definite diagnosis in jaundiced patients is important because all sulfonamides should be discontinued immediately if this form of chemotherapy appears to be responsible for the toxic complication. In individuals with jaundice due to other causes, sulfonamide medication, especially sulfadiazine, may be given without causing further damage to the liver parenchyma.<sup>1</sup>

In general, three forms of jaundice may accompany the use of the sulfonamide drugs. They may be classified as immediate, intermediate and delayed, depending upon the time of appearance of toxic symptoms, including icterus, following the initiation of therapy.

## IMMEDIATE JAUNDICE

The immediate form of toxic hepatitis secondary to sulfonamide medication is usually readily recognized. Within a period of one to three days from the time chemotherapy is started, jaundice appears. Prior to this, however, and usually within a few hours after the initial dose of sulfonamide, other toxic symptoms appear. These include nausea, vomiting, headache, chills, fever, burning of the eyes, and skin rashes. As the drug is continued the skin may become icteric and the urine dark with bile. Examination usually reveals an enlarged tender liver as well as various forms of skin rashes. The erythrocyte count and hemoglobin concentration are usually normal and the reticulocyte count is not elevated. The leukocyte count may be elevated or normal.

These patients give a history of previous ingestion of one of the sulfonamide drugs, and usually state that toxic symptoms occurred at that time.<sup>2</sup> An example of this form of hepatitis is reported below.

## CASE REPORT

A 45 year old married woman entered the Evans Memorial, Massachusetts Memorial Hospitals on September 14, 1940, because of chills, fever, and nausea of three days' duration. \* About eight months prior to admission she noticed that her afternoon oral temperature occasionally reached as high as 99.6 F.

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\* I am indebted to Dr. Chester S. Keefer for permission to report this case.

and, because of accompanying weakness, she reported to her physician. A diagnosis of endocervicitis was made and she was advised to enter the hospital for study. On July 28, 1940, she was admitted to another hospital where a blood culture revealed *Staphylococcus albus*. She was given sulfathiazole, 1 gram every 4 hours, beginning on August 10, 1940. The hospital record shows that the blood cultures became sterile and she was discharged on August 17. She continued to take sulfathiazole and, on August 24, two weeks after the institution of chemotherapy, she complained of photophobia, lacrimation and soreness and redness in several old scars around the left shoulder. Later a rash developed around the eyes and over the legs. Because of these findings the drug was discontinued by her physician with subsequent disappearance of all symptoms.

Because she continued to exhibit a low fever of about 99.2 F, on September 11, 1940, her physician again started sulfathiazole therapy. At 10 a.m., and 2 p.m. she took 1 gram. At 4 p.m. she developed a chill with a subsequent rise in temperature to 103 F. No further sulfathiazole was ingested until the following day when the dosage was increased to 1.5 grams. Again she took the drug at 10 a.m. and 2 p.m., but after the latter dose she developed a rigor, the temperature rising to 104 F. The next day she complained of a severe headache, aching in the joints, nausea, and vomiting, and noticed that her urine had become quite dark in color. She was hospitalized because it was thought she had developed hematuria.

The patient gave a history of tonsillitis in 1936 for which she had received sulfanilamide without the development of toxic symptoms.

On admission to the hospital the oral temperature was 100 F. There was a definite icteric tint to the skin and sclerae. There were no petechiae and no rash. The vessels of the conjunctivae and sclerae were injected. The nasal and pharyngeal mucous membranes appeared normal. The lungs were clear to percussion and auscultation. The heart was normal in size, no murmurs were heard. The liver edge was tender and extended 2 centimeters below the costal margin. The spleen was not felt.

Laboratory examinations included a total erythrocyte count of 4,000,000 per cubic millimeter, a hemoglobin of 71 per cent, and 4,200 leukocytes per cubic millimeter. The differential count on the blood smear showed 68 per cent neutrophils, 21 per cent lymphocytes, 2 per cent monocytes and 9 per cent eosinophils. There was bile in the urine as well as a trace of albumin.

*Clinical course.* The patient improved rapidly during the period of hospitalization. The icterus index which was 25 on September 16, 1940, had fallen to 4 on September 24. Bile was not detected in the urine after the third day in the hospital and the urobilinogen, which was present in a 1:20 dilution, decreased so that it was found only in undiluted urine. The reticulocyte count was 0.6 per cent on September 18 and the erythrocyte count and hemoglobin concentration did not change significantly. The Takata-Ara test was positive and there was a slight depression in the hippuric acid excretion test.

Because it was felt that this patient's illness was caused by sulfathiazole, she was given 0.5 gram on September 24, 1940. One hour later she developed a rigor, the vessels of the conjunctivae and sclerae became congested, she vomited twice, and complained of headache and soreness in the scars on the left shoulder. Four hours after the ingestion of the test dose of sulfathiazole the temperature reached 105 F. She was then given an intravenous infusion of 2000 milliliters of saline and the temperature rapidly returned to normal. No bile was detected in any of the urine specimens, but the icterus index gradually increased to 10 during the subsequent 24 hours. Urobilinogen was found again in dilutions of 1:20.

To summarize this case, the patient received an initial course of sulfathiazole and developed toxic symptoms two weeks later. These symptoms included nausea, vomiting, episcleritis, chills, fever, and skin rash. The drug was discontinued for approximately seventeen days and upon its resumption immediate toxic effects were exhibited. From the history it appears that jaundice developed two days after the first dose of the second course of sulfathiazole. When she had recovered, a test dose of 0.5 gram of sulfathiazole was administered. Again she developed fever, nausea, vomiting, and episcleritis. The icterus index rose from 4 to 10 and remained elevated for forty-eight hours. This patient's illness is an example of immediate jaundice due to previous sensitization to the sulfonamide drugs. There was little

evidence of hemolytic anemia although, from the studies recorded, it is not possible to state that some increase in the rate of destruction of the erythrocytes did not occur

### INTERMEDIATE JAUNDICE

This form of jaundice is associated with a mortality rate of 5 to 10 per cent<sup>3</sup> and is readily diagnosed as sulfonamide-induced since it is secondary to acute hemolytic anemia as well as to toxic hepatitis. The anemia usually becomes prominent enough to cause pallor, weakness, dyspnea, and nausea and vomiting in about two to five days after the institution of therapy,<sup>4</sup> although occasionally it may occur later.<sup>5-6</sup> Soon after the development of the acute hemolytic anemia, jaundice may appear. This is believed to be due not only to the very great destruction of erythrocytes, but also to some direct action on the liver cells.<sup>7</sup>

The clinical features of this form of jaundice are readily recognized. The patient invariably becomes critically ill during a period of a few hours, pallor is marked, the liver and spleen may be enlarged, and fever is usually present. Hemoglobin may appear in the urine. Later biliuria and urobilinogenuria are observed. The erythrocyte count and hemoglobin concentration are low, and a smear of the blood may show nucleated erythrocytes as well as variations in the size and shape of the cells. The reticulocyte count becomes markedly elevated. There may be spherocytosis and an increased hypotonic fragility during the acute phase of the disease.<sup>3</sup>

The total leukocyte count is usually markedly elevated, counts of 100,000 per cubic millimeter not being unusual. Immature cells are observed, as well as an increased number of eosinophils. The blood usually contains free hemoglobin as well as increased amounts of bilirubin.

It is apparent from this description that this form of sulfonamide jaundice should be easily recognized. It occurs most frequently following sulfanilamide medication although sulfathiazole, sulfapyridine and sulfadiazine occasionally cause acute hemolytic anemia and jaundice. When such toxic reactions occur, the drug must be stopped immediately and treatment, including blood transfusion, instituted.

### DELAYED JAUNDICE

Jaundice may appear ten or more days after the institution of sulfonamide treatment. Under these circumstances, the diagnosis of sulfonamide hepatitis may be difficult since jaundice may develop during the same period as a complication of the infectious process itself. Generally, however, in those patients whose jaundice is secondary to chemotherapy there are other associated toxic symptoms and physical signs.<sup>8</sup>

It is the usual experience that this form of hepatitis develops at about the time clinical improvement is anticipated. The temperature, which may have been normal or somewhat elevated, suddenly increases. In some instances it may be septic in type and associated with severe chills. Nausea, vomiting and epigastric discomfort may be prominent. Other symptoms of toxicity include headache, dizziness, photophobia, and aching of the joints. A distinctive feature which is of considerable aid in the establishment of the correct diagnosis is the appearance of

a rash which may be erythematous or, at times, exfoliative in character. Erythema nodosum is not infrequently observed in patients treated with the thiazole derivatives of the sulfonamides. Jaundice develops within a few hours or days after these other toxic symptoms have become manifest.

The physical signs and abnormalities in the laboratory examinations are similar to those found in patients developing the immediate form of jaundice. The liver and spleen may be enlarged and occasionally ascites may develop.<sup>2</sup> There may be a moderate anemia, especially if the reaction occurs after the ingestion of sulfanilamide. Usually there is an increase in the total leukocyte count, which may be marked when there is an extensive rash, and occasionally there may be leukopenia. The bilirubin content of the blood is increased and urobilinogen and bile are found in the urine. Impairment of liver function may be demonstrated by several tests.

Although most patients receiving sulfonamide therapy and developing the delayed form of jaundice conform to the above description, there are a few in whom the diagnosis is difficult. Toxic hepatitis apparently may develop several weeks after the cessation of chemotherapy. Garvin<sup>2</sup> reports an instance where jaundice and exfoliative dermatitis developed forty-three days after all sulfonamide medication had been stopped. In some patients jaundice appears as the only toxic manifestation of chemotherapy. One patient<sup>2</sup> developed jaundice sixteen days after sulfanilamide therapy was instituted and no other symptoms were recorded. In this instance the patient was being treated for chronic prostatitis, so that it would appear improbable that the hepatitis was secondary to the infectious process.

#### DISCUSSION AND SUMMARY

It is apparent from a review of the reported cases of hepatitis associated with sulfonamide therapy that it is usually possible to recognize this toxic manifestation. This is of considerable practical importance since, in every instance in which the sulfonamide is responsible for the jaundice, treatment with the drug should be discontinued and some other form of therapy, such as the antibiotics, instituted. If the jaundice is not secondary to the sulfonamide, therapy may be continued even in the presence of hepatitis secondary to the infection.<sup>1</sup>

Jaundice which appears during the first week of chemotherapy is usually associated with a previous history of ingestion of sulfonamides and accompanying signs of toxicity (immediate sulfonamide jaundice) or with acute hemolytic anemia and jaundice (intermediate jaundice). In either instance the diagnosis is not difficult because the clinical and laboratory abnormalities are characteristic. Finally, jaundice which occurs after ten days of chemotherapy is usually associated with other toxic manifestations, especially fever and various forms of rashes (delayed jaundice). Occasionally jaundice may be the only toxic manifestation of sulfonamide therapy and, in such patients, a definite diagnosis may be difficult.

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# BLOOD AND BONE MARROW IN INFECTIVE SUBACUTE AND CHRONIC ATROPHY OF THE LIVER

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## INTRODUCTION

NUMEROUS investigations have been made of the blood picture and bone marrow in hepatic diseases, particularly in cirrhosis of the liver. These have revealed that especially in cirrhosis of the liver, macrocytic anemia frequently develops. This can be accepted as a well-ascertained fact.<sup>4 5 21</sup>

It has further been shown that in liver diseases, especially in cirrhosis, changes also occur in the bone marrow. One sees rather frequently a slight or moderate increase in the number of erythroblasts, possibly a shift to the left in the erythroblasts, often a fair number of large forms, but no megaloblastic change in the marrow. There is also some increase in the plasma cells, and possibly also in the reticulum cells, with or without pigment. More rarely a slight myeloid hyperplasia or a slight eosinophilia are observed.<sup>6 7 12 13 15 20</sup>

As numerous cases of a special fatal form of subacute and chronic infective hepatitis have occurred in recent years and continue to do so here in Denmark, we have had the opportunity of examining the blood and bone marrow in this particular form of liver disease. The following notes deal with the nature of these cases.

In 1943 and 1944, subacute and chronic hepatitis began to occur in our clinic and in all other clinics in Denmark. Appearing suddenly, and so extensively and with such malignant symptoms, it produced the impression that an entirely new disease had arisen, and soon came to be known among both doctors and laity as "the malignant and dangerous jaundice." The most striking fact is that the patients were almost exclusively women of from 40 to 70 years of age.

The clinical picture is dominated in these cases by long-continued jaundice. It is, as a rule, only slight, but can be more intense or even severe. It varies somewhat in intensity, with a tendency gradually to subside, but in its place ascites and edema make their appearance in many cases in a few months' time, though usually in six months or a year, frequently at the stage when the jaundice has greatly decreased. After a longer or shorter interval, hepatic failure and death finally supervene. The prognosis is very grave, almost all the patients dying of the disease sooner or later.

At autopsy, a small contracted liver with isolated remains of liver tissue and an extensive development of coarse connective tissue is found, a picture previously described,<sup>2</sup> among others, in 1930 during a Swedish epidemic.

Preliminary reports have been published on the numerous cases,<sup>1 3 9 10</sup> and a more comprehensive joint report will appear in the near future. The disease must presumably be regarded as a form of acute infectious hepatitis, the extensive epidemic of which in Denmark reached its climax in 1944.

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## MATERIAL

The material which we have ourselves intimately studied is based on 24 women with subacute and chronic infectious hepatitis of the type described above. Their ages varied from 40 to 80 years. The history of the disease, when the investigation

TABLE 1—Blood Findings in 24 Cases of Infective Subacute and Chronic Atrophy of the Liver

No.	Name	Age	Sex	Duration of disease	Icteric index	Hemoglobin	Red cells	Color index	Mean diameter	Takata reaction	Sedimentation rate	Ascites edemas
				months		%			$\mu$			
1	A K L	57	♀	9	21	78	3.53	1.11	7.90	+++		+
2	O P	70	♀	3	16	85	4.24	1.02	7.78	+	9	—
3	M M A	66	♀	15	117	69	3.36	1.03	8.01	+++	62	+
4	O O K	57	♀	13	15	71	3.02	1.18	8.15	+++	27	+
5	K K H	70	♀	3	10	91	3.95	1.15	7.49	+++	30	—
6	E P P	68	♀	4	30	83	3.65	1.14	7.95	+++		—
7	A H.	62	♀	3	7	63	2.88	1.11	7.69	+		—
8	K N H	66	♀	1 (2)	12	93	4.65	1.00	7.77	+++	24	—
9	U M	51	♀	10	27	87	4.12	1.05	7.86	++		—
10	E J P	60	♀	6	15	80	3.54	1.13	7.64	+++	85	—
11	N B C	74	♀	13	21	78	3.56	1.10	8.62	+++		+
12	M K A	48	♀	6	24	65	4.20	0.77	7.77	+++	30	—
13	A K	79	♀	4	6	74	3.02	1.23	8.21	+++	48	+++
14	E C E S	71	♀	5	21	87	3.93	1.17	8.61	+++	15	+
15	E P	75	♀	5 (8?)	14	89	3.23	1.38	8.10	+++		+
16	S W O	68	♀	6	16	49	2.17	1.14	7.74		133	—
17	N M O N	56	♀	3	135	87	3.91	1.12	7.92	+++	82	—
18	H P	72	♀	2	81	96	3.86	1.24	8.01	+++	74	+
19	M A B	75	♀	12	27	95	3.91	1.22	8.10	+++	40	+
20	H M A	68	♀	?	15	87	4.26	1.02	8.19	+++	15	—
21	M E M C	63	♀	3	72	83	3.91	1.06	8.05	+++		—
22	A C	58	♀	5	11	76	3.21	1.19	8.04	++		+
23	A C C	80	♀	2 (1 year)	5	80	2.93	1.36	9.00	+++	14	+
24	B M L	69	♀	6	9	105	4.27	1.23	8.24	+++		+

NOTES Case 1 Died 1 month later Autopsy subacute atrophy of the liver Case 3 Died a few days later Autopsy subchronic atrophy of the liver Case 12 Died 2 months later Autopsy chronic atrophy of the liver Case 14 Died a few days later Autopsy chronic atrophy of the liver Case 15 Died a few weeks later Autopsy chronic atrophy of the liver Case 18 Died a few weeks later Autopsy chronic atrophy of the liver Case 23 Died 1 month later Autopsy chronic atrophy of the liver

took place, varied from 1-15 months duration. In other words, such an interval had elapsed since the jaundice was first noticed, but as, in a number of these patients, the disease developed rather insidiously, it had probably lasted as a rule longer than the specified period. Some of the patients had ascites and edema at the time the investigations were made, others developed these symptoms later. Some died shortly after the investigation, but most died later on.

Besides the above material, we have had access to other cases of the disease

approaching 150 in all, and from their records we have been able to collect further information about the blood changes

## METHODS

All the blood examinations were made by two well-trained technicians

The icteric index was determined by Meulengracht's method. The hemoglobin was estimated in an Autenrieth-Königsberger colorimeter, standardized to 100 per cent = 18.5 per cent oxygen binding capacity determined by Van Slyke's method.

Dilution of the blood for counting was done according to Ellermann's principle with separate pipets and mixing tubes, the blood corpuscles were enumerated in Zeiss counting chambers. The color index determinations were reckoned on the assumption that 100 per cent Hb corresponds to 5,000,000 red blood cells. With these methods and standards we find the color index in normal persons to be about 1.1.

The mean diameter of the red blood cells was determined by Gram's method, where they are measured in their own serum, so that one can be sure of avoiding the changes which may occur on drying or washing in foreign media. A drop of blood is sucked up by capillary attraction into a 5-10 cm. long, thin glass tube (capillary tube of about 1 mm. thickness). The tube is sealed at both ends and put aside for some hours. The blood has then clotted, and the serum has separated at the side of the clot. In this serum, free blood corpuscles are present in sufficient quantities for measurement and in suitable numbers so that measurement is not impeded by the corpuscles lying too close to one another. After the ends have been broken off the tube a small drop of serum is blown out into a counting chamber of half the usual height and is covered with a thin cover glass. The measurements are made with the help of an ocular micrometer and immersion lens. The diameters of 100 blood corpuscles at random are measured and the mean diameter calculated. Mulberry shaped, spherical and other abnormally shaped blood cells are avoided.

The Takata reaction was performed by Jezler's modification. The blood sedimentation rate was determined by Westergren's method.

In the smear preparations of the sternal punctures, a differential count of 200 cells was made. Further more, in every case a section of the coagulum of the sternal puncture after embedding in paraffin was examined.

The investigations of the sternal punctures were all made by one of us (H. G.)

## RESULTS

*Blood.* In table 1, the age, sex, duration of the disease and the results of the blood examinations are given for those patients we ourselves thoroughly investigated.

The icteric index varied from 7 to 135, it often became decreased as the cirrhosis progressed. The Takata reaction in most of the patients was strongly positive (+++), but in some it was weaker (+ or ++). In one case it was negative, but it is possible that the diagnosis was wrong. The blood sedimentation rate was increased in all the cases investigated.

The hemoglobin values varied from 50 to 105 per cent, red blood cells, from 2.17 millions to 4.65 millions. In most cases, there was some anemia. The color index varied from 0.8 to 1.36. It was usually around 1.1, which with our standards corresponds to the normal, in a few cases it was perceptibly diminished. On microscopic examination of the stained dry preparation, neither oval cells nor large ones of the nature of megalocytes were observed. From the other more extensive material we have had access to, we have collected 141 determinations in 75 patients. Hb varied from 58 to 104 per cent, R. B. C. from 2.5 millions to 5.1 millions, color index from 0.8 to 1.34. In figure 1 the Hb values are plotted against the red cell values, and the color index given. It will be seen that there is a marked tendency to a moderate degree of anemia, the bulk of the values lying between 60 and 80 per

cent Hb, and between 3 and 4 million red cells. The color indices are grouped around 1.1

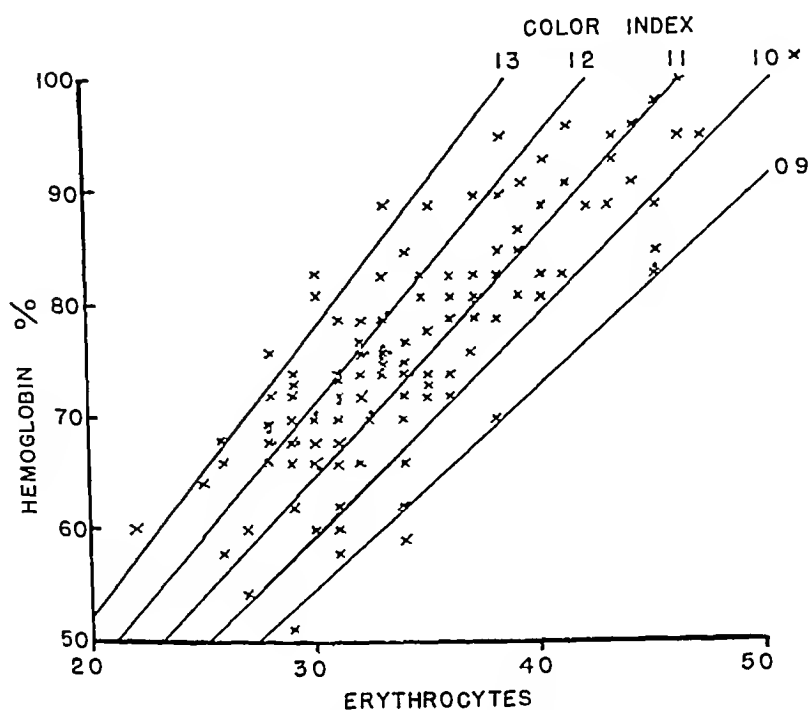


FIG. 1. HEMOGLOBIN RED CELLS AND COLOR INDEX IN 141 DETERMINATIONS IN 75 CASES OF INFECTIVE SUBACUTE AND CHRONIC ATROPHY OF THE LIVER

Lines are continuations from a common point representing zero for both hemoglobin and erythrocytes

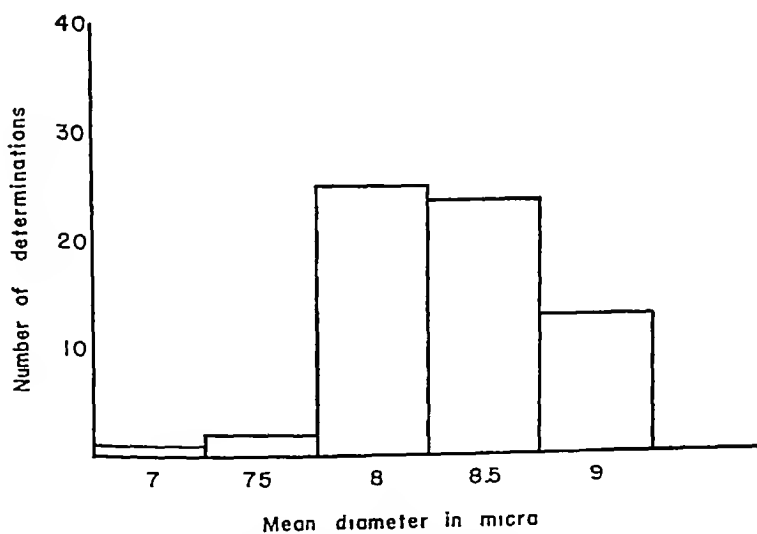


FIG. 2. MEAN DIAMETER OF RED CELLS IN 64 MEASUREMENTS IN 52 CASES OF INFECTIVE SUBACUTE AND CHRONIC ATROPHY OF THE LIVER

In the closely studied cases, the mean diameter of the red blood cells varied from  $7.64\mu$  to  $9.0\mu$ . From the other material we collected 64 measurements in 52 patients, there was about the same variation. The values are given in table 2 and figure 2.

It will be seen that over 50 per cent lie above  $8\ \mu$ . With the same technic, Gram<sup>8</sup> found values fluctuating between  $7.7\ \mu$  and  $8.0\ \mu$  in normal persons. Jørgensen and Warburg<sup>11</sup> found roughly corresponding limits. In our laboratory also, the normal values found are almost identical with those of Gram. On the basis of these facts it can be asserted that there is a very decided tendency towards an increase in the mean diameter of the red cells in patients suffering from subacute or chronic atrophy of the liver, in No. 23 it reached as much as  $9\ \mu$ .

TABLE 2.—Mean Diameter of Red Blood Cells in 64 Measurements in 52 Patients

$\mu$ Number	7 1	7-7.5 2	7.5-8 35	8-8.5 23	8.5-9 13
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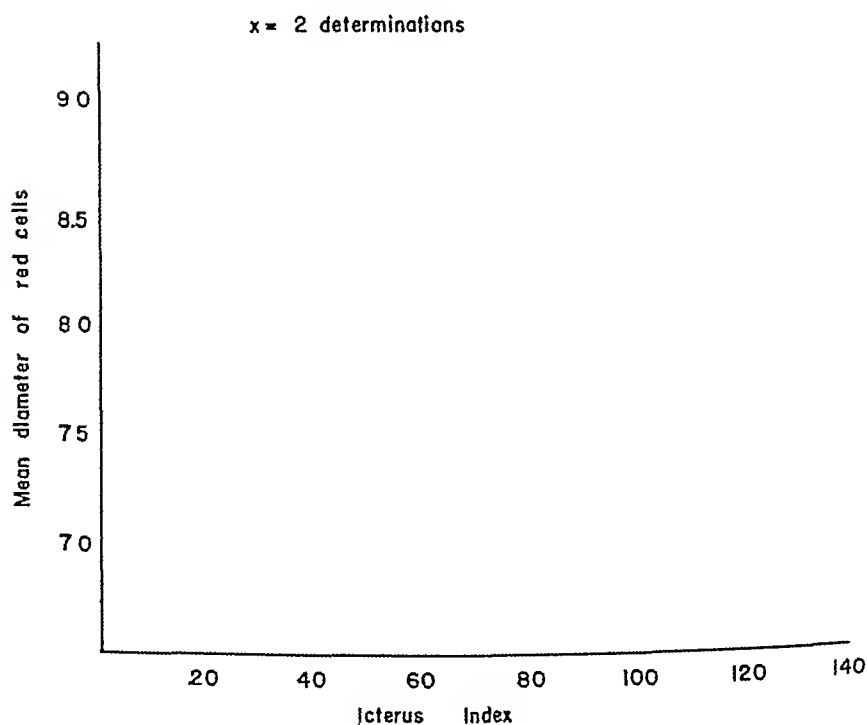


FIG. 3. MEAN DIAMETER PLOTTED AGAINST ICTERIC INDEX

In figure 3, the mean diameter is plotted against the icteric index. It will be observed that there is no correlation between the two.

In figure 4, the mean diameter is plotted against the number of red cells. There is no correlation between the mean diameter and the degree of anemia.

In figure 5, the mean diameter is plotted against the color index. Again no correlation can be detected.

The number of white blood corpuscles varied between 1700 and 12700 in 129 counts in 81 patients (table 3 and figure 6). The last value was quite an isolated one. There was a distinct tendency to low counts, over 50 per cent of them being under 4000. Unfortunately, differential counts were done in only a few cases, but as far as they went no definite deviation from the normal was found.

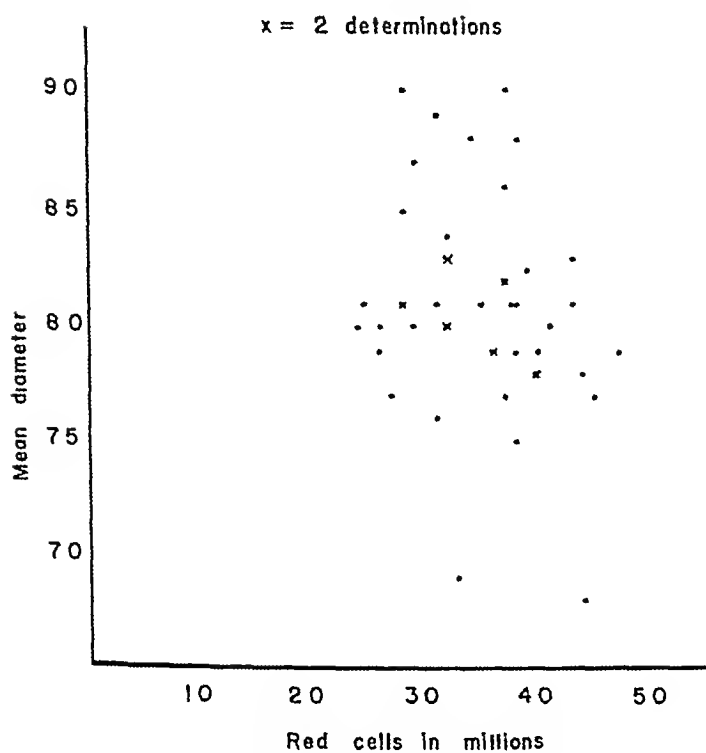


FIG 4 MEAN DIAMETER PLOTTED AGAINST RED CELLS

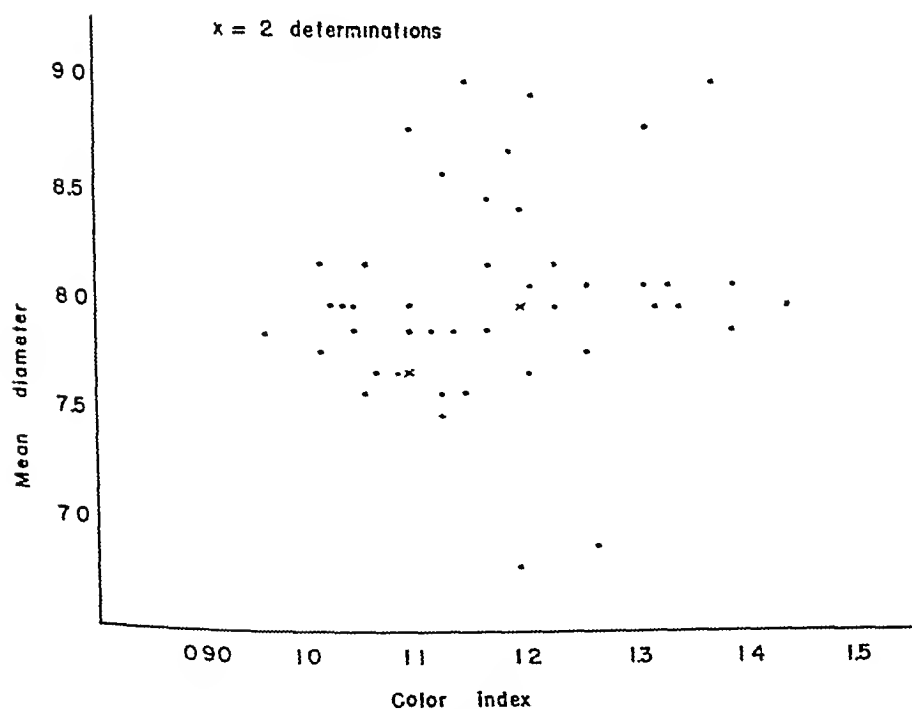


FIG 5 MEAN DIAMETER PLOTTED AGAINST COLOR INDEX

The blood platelets were counted in only a few of the cases. There appeared to be tendency towards low values.

*The bone marrow* The results of the sternal punctures are given in table 4 As a basis for comparison, the normal values found by Gormsen<sup>7</sup> in a material of 50 normal adults are given in the first column

A comparison with the normal values indicates that in the neutrophile granular cell series there were no changes, no shift to the left, no myeloid hyperplasia and no eosinophilia Hemocytoblasts, lymphocytes, megakaryocytes and reticulum cells exhibited normal appearances, but there were certain abnormalities in the erythroblasts and plasma cells

Four patients had over 30 per cent erythroblasts, 8 patients had from 22 to 26 per cent, while the remaining 12, therefore half the patients, had below 20 per cent

TABLE 3 —Number of Leukocytes in 129 Counts in 81 Patients

Leukocytes	1000— 2000	2000— 3000	3000— 4000	4000— 5000	5000— 6000	6000— 7000	7000— 8000	8000— 9000	9000
Number	3	25	44	24	23	4	4	1	1

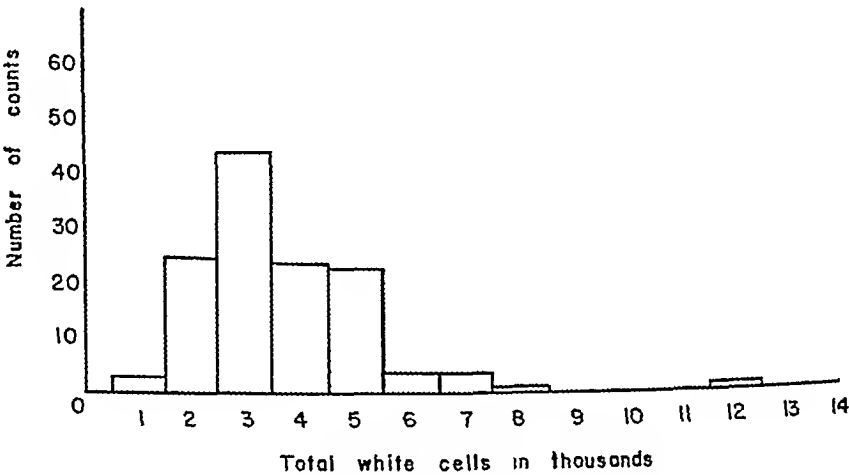


FIG 6 NUMBER OF WHITE CELLS IN 129 COUNTS IN 81 CASES OF INFECTIVE SUBACUTE AND CHRONIC ATROPHY OF THE LIVER

In none was the number of erythroblasts found to be lowered In one patient only (No 22) was there a shift to the left an the erythroblasts, but it was very pronounced No definite macrocytosis of the erythroblasts could be detected in any of the cases Micrometric investigations of the size of the erythroblasts, which normally vary considerably, were not undertaken Megaloblasts were not observed in any of the cases

With regard to the plasma cells, they were 4 per cent in 3 patients, 3 per cent in 6 and below 3 per cent in the other 15, that is to say, in 9 of the patients there was a slight increase in the plasma cells, since the maximal value of 3 per cent for the normal number of plasma cells is very high

Microscopic sections of the bone marrow in 8 patients (Nos 1, 2, 3, 8, 11, 12, 17, 18) showed slight to moderate hyperplasia of the marrow, but in the others the cell content was normal The hyperplasia seemed to depend in part on a striking increase in the erythroblasts, and also on a slight augmentation of all the other cell

forms, so that the state of the marrow seemed to point to a slight increase in the functioning of the bone marrow with greatest emphasis on erythropoiesis

TABLE 4—Results of Sternal Puncture in 24 Cases of Infective Subacute and Chronic Atrophy of the Liver

	Normal values		No. of patient														
			1	2	3	4	5	6	7	8	9	10	11	12			
	%	%															
Neutroph segment	19	10	-25	18	13	8	12	19	18	20	18	24	22	27	36		
Neutroph nonsegment	6	2	-10	4	5	6	6	6	5	5	8	6	6	5	5		
Neutroph metamyelocytes	15	8	-20	14	13	21	16	21	16	19	14	18	17	12	10		
Neutroph myelocytes	12	7	-18	9	11	13	15	12	11	12	16	13	11	11	9		
Neutroph promyelocytes	4	2	-7	5	6	7	6	6	6	6	6	6	5	4	3		
Eosinoph matures	1	0	-3	5	1	1	1	2	1	1	3	2	2	1	1		
Eosinoph immatures	2	0	5-4	2	5	4	1	4	3	3	4	4	2	1	2		
Basoph granulat	1																
Hemocyto blasts	1	0	3-2	5		1	1				1			1			
Lymphocytes	15	5	-25	11	8	4	11	8	10	12	6	9	10	18	12		
Monocytes	1	0	-3														
Plasma cells	1	0	-3		3	3	3	2	1	2	0	5	2	1	10	5	2
Normoblasts	10	6	-17	21	17	19	13	9	15	10	12	6	11	10	9		
Polychrome erythroblasts	6	3	-9	6	10	7	9	7	7	7	6	5	7	4	5		
Basophile erythroblasts	3	1	-4	4	5	4	4	2	4	3	2	3	3	2	3		
Megakaryocytes	1																
Reticulum cells	3	0	5-9	2	3	2	2	4	2	2	5	2	3	2	3	5	3

	Normal values		No. of patient													
			13	14	15	16	17	18	19	20	21	22	23	24		
	%	%														
Neutroph segment	19	10	-25	16	18	14	12	13	17	15	18	32	9	19	15	
Neutroph nonsegment	6	2	-10	5	7	7	7	5	8	7	7	6	4	5	6	
Neutroph metamyelocytes	15	8	-20	16	16	15	18	16	15	15	14	9	18	14	18	
Neutroph myelocytes	12	7	-18	13	12	14	13	14	11	12	11	7	10	13	13	
Neutroph promyelocytes	4	2	-7	6	6	6	6	7	5	6	6	5	5	7	7	
Eosinoph matures	1	0	-3	5	1	2	1	1	1	1	1	1	1	1	1	
Eosinoph immatures	2	0	5-4	2	3	3	2	2	2	2	3	2	2	2	2	
Basoph granulat	1															
Hemocyto blasts	1	0	3-2	5	1				0	5	1			1	1	
Lymphocytes	15	5	-25	8	9	12	12	10	15	13	16	22	9	21	15	
Monocytes	1	0	-3													
Plasma cells	1	0	-3		3	1	2	1	4	4	3	2	2	3	2	4
Normoblasts	10	6	-17	15	11	15	15	13	11	10	9	6	9	7	9	
Polychrome erythroblasts	6	3	-9	7	8	7	6	7	5	7	6	4	13	4	4	
Basophile erythroblasts	3	1	-4	4	4	4	3	5	2	5	4	2	14	2	2	
Megakaryocytes	1															
Reticulum cells	3	0	5-9	3	3	4	4	3	3	5	3	3	2	3	2	3

#### COMMENT

In the blood examinations, i.e., determination of Hb per cent, R B C, color index and direct microscopy, no changes such as those found in pernicious ane



were observed. In conformity with this no sign of megaloblastic transformation of the red bone marrow was seen in any of the bone marrow punctures. We have in some cases attempted to influence the anemia by liver injections, but there was no reticulocyte reaction and no rise in the number of red blood cells.

On the other hand, a marked tendency to an increase in the mean diameter of the red blood cells was observed, which in a few cases assumed very high proportions. Gram,<sup>8</sup> in 1883, in his measurements of the diameter of the red cells in various diseases demonstrated that the mean diameter in 'ikterus catarrhalis,' that is, hepatitis, is increased. Determined in their own serum, the values lay between  $8\ \mu$  and  $8.5\ \mu$ , in one case with cirrhotic changes it was  $8.9\ \mu$ . This finding was later confirmed by others<sup>11-14</sup> using the same method. The increase in the mean diameter in such cases of liver disease has been attributed to changes in the plasma, possibly to the presence of the salts of bile acids. The fact that in our bone marrow investigations only doubtful macrocytosis of the erythroblasts was demonstrated, and at all events no megaloblastic change in the bone marrow, seems to support the theory that the macrocytosis may at least partly be due to a change in the plasma with consequent swelling of the blood corpuscles. Since there is no correlation between the degree of jaundice and the increase in the mean diameter, the latter cannot depend upon the bilirubinemia but must be caused by other substances in the plasma (bile acid salts?) The question needs a more elaborate investigation.

The tendency to an increase in the plasma cells of the bone marrow must undoubtedly be connected with the plasma protein changes causing the positive Takata reaction, which are characterized by an alteration in the albumin/globulin ratio, decrease in the albumin fraction and increase in the globulin fraction.

### CONCLUSIONS

In the observed cases of subacute and chronic infectious atrophy of the liver, of the special type which occurs at the present time in Denmark, a moderate degree of anemia of a hypochromic, normochromic or hyperchromic type was found in the majority of the cases. The color index showed a fairly uniform distribution around the normal value 1.1. There was nothing in the blood picture that resembled true pernicious anemia.

Measurements of red blood cells in their own serum revealed an increase in the mean diameter, often considerable, in over half the cases.

The blood picture with respect to the white corpuscles showed a pronounced tendency in the direction of leukopenia.

In some of the cases, sternal punctures showed the presence of a more or less advanced erythroblastosis, but no really definite macrocytosis of the erythroblasts was observed, and megaloblastic erythropoiesis occurred in none of the cases. A slight increase in the plasma cells was seen in some cases. Sections of the bone marrow, in a number of cases, showed moderate hyperplasia of the marrow.

In no case, either in the blood or bone marrow, could changes be demonstrated which would suggest nonstorage of the material necessary for the maturation of the red blood cells. The increase in the mean diameter of the red blood cells measured in their own serum, which was observed in some cases, is not due to megaloblastic transformation.

blastic erythropoiesis, but possibly to the serum content in substances which affect the osmotic condition of the blood corpuscles

The plasma cell increase in the bone marrow that was found in some of the cases, is presumably connected with the hyperglobulinemia which is a very common accompaniment of chronic hepatitis

The blood and bone marrow changes in our patients thus do not seem to differ from the blood and marrow changes hitherto recognized in chronic hepatitis and liver cirrhosis

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# MYELOFIBROSIS ASSOCIATED WITH TUBERCULOSIS

## A REPORT OF FOUR CASES

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AND WALTER H. NADLER, M.D.

### INTRODUCTION AND REVIEW OF THE LITERATURE

**M**YELOFIBROSIS is a disease in which the normal blood-forming elements of the bone marrow are replaced by fibrous tissue with compensatory extramedullary hemopoiesis arising in other organs of the reticulo-endothelial system. Clinically, it is characterized by pains in the long bones, back or abdomen, progressive weakness, pallor and subsequent loss of weight. The spleen, liver and sometimes the lymph nodes become enlarged and a refractory anemia of the myelophthisic type develops. Immature leukocytes with or without an increase in total count and frequently the platelet count is either decreased or increased. Bone marrow studies show hypoplasia, usually an increase in the megakaryocytes and eventually, fibrosis. The onset of the disease is insidious and it may last from a few months to years, depending upon the degree of compensation, but the eventual outcome is fatal.

Myelofibrosis was first described by Heuck<sup>1</sup> in 1879, and since that time approximately 100 cases have been reported in the literature under a great variety of titles, the most common of these are "leuco-erythroblastic anemia,"<sup>2</sup> "chronic non-leukemic myelosis"<sup>3</sup> and "myelofibrosis associated with a leukemoid blood picture."<sup>4</sup> The name "myelofibrosis" was first applied to this disease in 1937 by Mettler and Rusk.<sup>4</sup> In 1944, Erf and Herbut<sup>5</sup> contributed materially to this subject by their extensive review of the literature and classification of myelofibrosis as either a primary disease or a disease secondary to such conditions as benzene or fluorine poisoning, irradiation and malignant extension. No mention is made of the possible etiologic role of tuberculosis, although such a relationship had been previously suggested. Among 91 cases of myelofibrosis reported in the literature, there were 7 cases with definite evidence of active tuberculosis. A summary of the findings in the 7 cases is recorded in table 1. The first American to report a case of myelofibrosis was Donhauser<sup>6</sup> (case A) in 1908. His case was found to have an active tuberculosis involving the mesenteric lymph nodes and he proposed a toxic etiology for the primary marrow disease. One of the 5 cases reported by Dyke<sup>7</sup> (case B) in 1924 had military tuberculosis, the remaining 4 had other bacterial diseases with bone marrow involvement, and he suggested a disseminated bacteremia as an etiologic factor in this disease. Krasso and Northnagel<sup>8</sup> (case C) in 1925 found atypical tuberculous lesions in their case which they believed were caused by avian tubercu-

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losis Emile-Weil, Chevallier and Sec<sup>9</sup> (case D) in 1933 proposed the possibility of a tuberculous etiology for this disease, and in 1935 Hugonot and Sohier<sup>10</sup> reported a case (case E) of myelofibrosis associated with tuberculosis and agreed with this etiologic relationship. Stone and Woodman<sup>11</sup> (case F) in 1938 reported a case of myelofibrosis with tuberculosis. They point out the frequency in which tuberculous lesions are found in diseases of the reticulo-endothelial system. Carpenter and Flory<sup>12</sup> in 1941 concluded that the tuberculosis in their case (case G) was a coincidental terminal disease.

The purpose of this paper is to review in detail the clinical picture and autopsy findings of 4 cases of myelofibrosis associated with generalized tuberculosis and to discuss the pathogenesis, diagnosis and treatment of this syndrome. We have observed 5 cases with idiopathic myelofibrosis which will not be reviewed in detail.

TABLE 1—Summary of Cases from the Literature of Myelofibrosis Associated with Tuberculosis

History			Physical Findings				Laboratory Examination			Clinical Course	Autopsy				
Case	Age, Sex	Weight loss	Fever	Splenomegaly	Hepatomegaly	Lymphadenopathy	Anemia	Leukemoid reaction	Thrombocytes	Duration of illness months	Tuberculosis		Extramedullary hemopoiesis	Marrow fibrosis	Fibrosis of other organs
											Focal	Miliary			
A	M, 58	++	++	+++	+	—	++	+	?	4	—	+	+	++	+++
B	M, 34	?	++	?	?	++	+	?	?	6	—	+	?	+	?
C	M, 45	++	+++	++	+	+	+	+	?	24	—	+	+	++	?
D	?, f	?	?	+	+	+	++	++	?	?	—	?	+	++	+
E	M, 64	++	+++	+++	++	+	++	+	?	26	—	+	+	+	+
F	F, 43	++	?	++	+	+	++	+++	?	12	+	—	+	++	+
G	M, 33	++	+++	++	++	+	++	+++	+++	40	—	+	+	+++	++

? = not recorded

in this communication but serve for general comparison with the tuberculous group.

## REPORT OF CASES OF MYELOFIBROSIS ASSOCIATED WITH TUBERCULOSIS\*

### CASE 1

C B, a 39 year old housewife, was admitted to the hospital June 7, 1941, complaining of pallor, weakness, dizziness, a skin rash and fever.

*Family history* Irrelevant.

*Past history* Amenorrhea had been present for nine years. She had pneumonia at 36 years of age and again at 38. She was occasionally mildly jaundiced and bruised very easily during the last few years.

*Present illness* The pallor, weakness, and dizziness had been present for the previous thirteen years accompanying episodes of unexplained anemia. These episodes recurred with increasing frequency and persisted during the previous eighteen months. There was no bleeding from any of the orifices. She failed

\* Only contributory clinical and pathologic findings are described.

to respond to either liver or iron and received whole blood several times. Following a splenectomy and subsequent phlebitis, she had a daily afternoon temperature elevation, occasionally reaching 101 F. The skin rash started on the left leg three weeks before hospital entry, it then extended to the right leg and both arms. The rash was maculopapular at first, later indurated and finally tender.

*Physical examination* The pulse was 110 per minute, blood pressure, 116/76, respirations, 24 per minute, temperature, 101.6 F, and weight, 99 pounds. She appeared somewhat emaciated and chronically ill. Over the hands, elbows, and knees the skin had a dusky appearance. An eruption over the legs and arms varied from small, red maculopapules to reddish purple, eczematoid, tender, indurated, nodular lesions distributed mainly over the extensor surfaces. The sclerae were white and the mucous membranes pale. There were a few small cervical, axillary and inguinal lymph nodes palpable. Occasional coarse rales were heard over the base of the left lung posteriorly, with slight dullness in this same region upon percus-

TABLE 2.—Representative Peripheral Blood Pictures from the Cases of Myelofibrosis with Tuberculosis

Case	Date	Erythrocytes (mil lion per cu /mm)	Hemoglobin (Gm / 100 cc)	Leuko-cytes (per cu / mm)	Thrombo-cytes (per cu / mm)	Myeloblasts	Promyelocytes	Neutrophils myelo cytes	Metamyelocytes	Bands	Segmented	Eosinophils	Basophils	Lymphocytes	Monocytes	Broken cells	Normoblasts/100 WBC
C B	1/31/40	1 58	6 0	3,400						44	29	2		25			2
	4/23/40	1 59	6 0	9,000							54	4		40	2		53
	6/7/41	2 14	7 0	6 050	138,000			2	4	16	45			33			36
W M	11/10/43	2 40	8 5	8 400													
	12/20/43	2 00	5 8	11,800	251,320	5		6	6	29	18	2		30	4		6
	3/13/44	2 76	7 5	3,500	Dec	4				30	18		2	44	2		
W D	1/3/46	4 42	13 2	2 850						18	61			16	5		
	2/1/46	4 70	14 0	4,700	247,000				2	27	33			25		13	
	5/16/46	3 93	10 5	1,700	133 350					48	32			20			
	6/1/46	3 50	9 5	2,100	208,320				3	13	67			8	9		2
M B	10/19/44	3 80	8 1	17,600					3	5	59			28	5		
	6/30/45	1 90	4 35	60,000	500,000			10	8		14			22	42	4	31
	8/13/45	3 50	8 7	115,000	700,000	60	13	6	8	4	5			2		2	

cussion. There was a tachycardia and a coarse systolic murmur was audible over the entire precordial region. The abdomen was soft and not tender, the liver was not palpable.

*Laboratory examination* The urinalysis showed no sugar and a trace of albumin. The sediment contained 3-5 erythrocytes per high power field and on one occasion hyalin and granular casts. The Wassermann and Kahn tests were negative. A fractional gastric analysis showed 48 units of free HCl and 70 units of total acid. The BMR was plus 6 per cent. The Van den Berg test was 0.8 mg per cent and the icterus index was 9 units. Other blood chemistry studies were normal. A total of ten stool examinations for blood were found negative. Agglutination tests for typhoid, paratyphoid, dysentery and brucella were negative. Sputum cultures revealed no acid-fast bacilli but many gram-positive diplococci. Blood cultures and fecal cultures were negative. X-ray examination of the chest was normal on 6/7/41, but one week later there was evidence of fluid at the base of the left lung. Hematologic studies (table 2) showed a pronounced normocytic, normochromic anemia with numerous nucleated erythrocytes and a few immature granulocytes in the peripheral blood. There was a moderate variation in the size and shape of the erythrocytes. The reticulocyte count was 1.3 per cent and the platelets were reduced to the lower limit of normal. The bone marrow (table 3) was hypoplastic and revealed a maturation arrest of the erythrocytic series and an increase in the number of megakaryocytes. The red cell fragility test

was normal on two occasions. The spleen, surgically removed on 8/28/40, was reported essentially normal. A skin biopsy of a nodule from the left arm on 6/10/41 was diagnosed as possible erythema nodosum. Acid fast organisms were found in sections made from paraffin blocks containing sediment from pleural fluid. A guinea pig inoculated with pleural fluid was found to have epithelioid tubercles of the lungs, liver and spleen.

**Clinical course.** The patient had a very stormy course throughout her hospital stay with spiking daily afternoon temperature elevations, sometimes to 106 F, preceded by chills. She became dyspneic, irrational and very restless and expired on 7/3/41.

#### Necropsy

**Thoracic cavity.** The left pleural cavity contained 1000 cc of bright red fluid. Both lung bases were adherent to the diaphragm and mediastinum by firm fibrous adhesions. The pleural surfaces were studded with pin-head sized grey, raised tubercles. Microscopically, the alveoli in the left lung base were filled with a fibrinous exudate. The tiny tubercles showed a central necrosis with almost no surrounding cellular reaction.

TABLE 3—Differential Sternal Marrow Counts on Cases of Myelofibrosis with Tuberculosis

Case	Date	Total count	Myeloblasts	Promyelocytes	Neutrophils myelocytes	Metamyelocytes	Bands	Segmented	Eosinophils	Basophils	Lymphocytes	Monocytes	Broken cells	Pronormoblasts	Normoblasts (all types)	Megaloblast	Megakaryocytes	Myeloid/erythroid ratio
C B	3/ 5/40		3		15		20	6			6				44	6	Inc	1/1
W M	12/20/43	115,000	8	1	2	2	6	4			3		2	23	40	6	Inc	1/3
	3/13/44	12,500	31		2	1	12	8			31	2		2	6		Dec	11/1
	3/28/44	6,250	10				4		6	48		2	2	4		14	Inc (5)	4/1
W D	2/ 1/46	176,000	3	3	5	8	20		1	5					55		Inc	1/1
	5/16/46	45,000		7	8	18	25	1	3	2	5			3	28		Inc	2 4/1
M B	7/ 9/45		16	15	4	3	4	9	4	10			2	33		Inc	2/1	

Heart The greater vessel

**Heart.** The greater vessels and epicardium were covered with the same tiny tubercles seen on the lungs. Histologic study showed an occasional necrotic focus in the myocardium.

**Liver.** The liver was enlarged and an occasional tubercle was seen on the capsule and cut surface. The sections showed thickening of the capsule with increased intercellular connective tissue. Numerous tiny areas of necrosis were seen in the parenchyma and each was surrounded by a zone of immature blood cells. A few small foci of extramedullary hemopoiesis containing occasional megakaryocytes were seen.

**Pancreas.** Grossly, the pancreas appeared normal but microscopic studies revealed pronounced interacinar fibrosis and numerous small necrotic tubercles, a few of which were surrounded by immature blood cells.

**Lymph nodes.** The hilar, retroperitoneal and abdominal lymph nodes were all grossly enlarged. Many showed discrete pin head sized, grey tubercles on their cut surfaces. Microscopically the normal architecture of the glands was completely destroyed and replaced by proliferating granular atrophic fibrous tissue occasionally surrounding small foci of extramedullary hemopoiesis. A few hyperchromatic, multi nucleated cells resembling megakaryocytes were seen. Many of the nodes showed small foci of tuberculous necrosis and a rare Langerhans giant cell.

**Bone marrow.** The marrow from the sternum and vertebrae appeared dark red and dry. Histologically the sections showed a fibrous tissue replacement of the marrow cavity leaving only a few hemopoietic tissue and no fat. The megakaryocytes were increased in number.

to respond to either liver or iron and received whole blood several times. Following a splenectomy and subsequent phlebitis, she had a daily afternoon temperature elevation, occasionally reaching 101 F. The skin rash started on the left leg three weeks before hospital entry, it then extended to the right leg and both arms. The rash was maculopapular at first, later indurated and finally tender.

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# MYELOFIBROSIS ASSOCIATED WITH TUBERCULOSIS

*Bacteriologic examination* All the sections were stained by the Ziehl-Neelson technic and the necrotic foci found filled with acid-fast organisms

*Pathologic diagnosis* Generalized miliaty tuberculosis, extramedullary hemopoiesis in the liver and lymph nodes, fibrosis of the liver, pancreas and bone marrow

## CASE 2

W. M., a 50 year old lawyer, was first admitted to the hospital November 10, 1943, complaining of angular pain, intermittent claudication and pallor

*Family history and past history* Irrelevant

*Present illness* For two years the patient had noted viselike pain in his chest upon exertion. During the last thirteen months, shortness of breath came with the chest pain, both were relieved by rest. Simultaneously, he developed cramplike pains in the calf of his left leg, brought on by walking. An electrocardiogram in February 1943 showed no significant change. These symptoms became progressively worse and he subsequently noticed that he was becoming fatigued and pale.

*Physical examination* The temperature was 99.6 F, blood pressure, 120/60, pulse, 96 per minute, respirations, 18 per minute, and weight 178 pounds. The lymph glands were normal. There was no cardiac abnormality. The lungs were clear except for inspiratory wheezes over both apices. The abdomen was soft, and the liver, spleen and kidneys were not felt.

*Laboratory examination* The urine was repeatedly negative. The Wassermann and Kahn tests were negative. The total serum proteins, albumin/globulin ratio and icterus index were all normal. X-ray examination of the chest showed the lung fields to be clear and the heart shadow less than 10 per cent enlarged. Roentgenologic examination of the gall bladder and digestive tract revealed no pathology. The electrocardiogram showed only a left axis deviation. The BMR was minus 7.5 per cent. Repeated blood cultures, stool cultures for enteric pathogens, agglutination tests for typhoid, paratyphoid, and a brucella skin test were all negative. The blood studies (table 2) revealed a moderate normochromic, normocytic anemia with immature leukocytes and normoblasts in the peripheral blood. The reticulocyte count was normal and the platelets were slightly decreased in number. The sternal marrow (table 3) showed an initial hyperplasia with increased erythropoietic activity followed by a marked hypoplasia with an increase in megakaryocytes.

*Clinical course* Seven days after entry into the hospital the patient was seized with excruciating precordial and epigastric pain with tenderness over the gall bladder, all of which disappeared two days later. During this stay, he had a slight fever for the first two days, again on the seventh day and again on the tenth, eleventh, and twelfth days. He was discharged November 27. He was next seen December 14 as an outpatient complaining of increased weakness and had a decline in the erythrocyte count. He was readmitted to the hospital December 29 for two days and given 400 cc. of whole blood. He continued under ambulatory care without response and was readmitted to the hospital on February 2, 1944. There was no fever at this time. A slight systolic murmur was heard over the apex of the heart. The lungs were normal. The liver was normal in size and the spleen was felt for the first time, extending two finger breadths below the left costal margin. There was a slight generalized lymphadenopathy. He was given two transfusions of 500 cc. of whole blood each and discharged after twenty-four hours. On February 17, while the patient was ambulatory, the spleen was found to be the same size. The liver was now found three finger breadths below the right costal margin and the anemia continued to progress. His last hospital entry was February 23. In addition to previous complaints, he had painful defecation and gross blood in the stools for ten days. His temperature on admission was 100.6 F, pulse, 68 per minute, respirations, 20 per minute, and weight, 164 pounds (a loss of 14 pounds since his first admission). The liver and spleen were palpable as previously. A proctoscopic examination was done under caudal anesthesia and a diagnosis of ulcerative proctitis was made. Four days later, an indurated region just outside the sphincter ruptured and spontaneously drained mucopurulent material. He was given numerous transfusions of whole blood for profound anemia but failed to maintain a satisfactory hemoglobin and red cell count level. The sternal puncture (table 3) on February 28 showed a pronounced hypoplasia of the marrow with a marked increase in the number of megakaryocytes. The temperature rose daily to over 100 F, and after the seventeenth hospital day, and the patient expired two days later, April 2, 1944.

*Necropsy*

*Lungs* Neither gross nor microscopic miliary nodules were described. The sections showed emphysema and focal hemorrhages surrounded by a few hyperchromatic multi-nucleated cells. Pleural thickening was also seen.

*Mediastinum* A firm nodular mass measuring 6 by 3 by 2 cm. was found in the right mediastinum lying just behind the superior vena cava, superior to the root of the right lung and lateral to the arch of the aorta. This mass cut with ease revealing a bulging, pinkish surface. Several grey-green nodules 1-2 cm. in diameter were seen on the cut surface and several small cystic structures contained purulent material. Microscopic studies of this mass revealed confluent lymph nodes almost completely replaced by granulomatous tissue. There were central zones of caseation and necrosis surrounded with varying numbers of epithelioid cells and lymphocytes, all of which were encased in a fibrous tissue. A few multinucleated cells similar to those described in the lung were seen. No Langhans-type cells were present. Several large nerve bundles coursed through the dense fibrous tissue.

*Abdominal cavity* Firm fibrous adhesions were found about the gall bladder and the upper portion of the colon.

*Liver* The liver was enlarged (2900 Gm.) and small yellowish nodules were seen beneath the capsule and on the cut surface. Histologic examination showed the sinusoids distended and filled with blood. A fibrous tissue and lymphocytic infiltration was noted about the portal triad. Small nodular foci were seen surrounded by a few epithelioid cells, lymphocytes, and plasma cells. An occasional megakaryocyte was seen. Pronounced fibrous tissue infiltration and hyalinization in the regions adjacent to these atypical tubercles were constant findings.

*Spleen* The spleen weighed 550 Gm. and several small nodules were palpated beneath the capsule. Microscopically, small foci of caseous necrosis were surrounded by a few round cells. In one of these a single Langhans-type giant cell was seen. The sinusoids were distended and filled with blood and pigment. Small lymph follicles remained. Throughout the pulp, numerous large cells with large oval or indented, hyperchromatic nuclei were found. Many resembled megakaryocytes. Hemopoiesis was not pronounced but the number of immature myeloid cells indicated the presence of this function. The capsule was thickened.

*Adrenals* The gross features of this organ were not unusual but microscopically both the medullary and cortical layers showed focal necrosis and rather extensive dense fibrous tissue replacement and hyalinization.

*Gastro-intestinal tract* The only finding of significance in the gastro-intestinal tract was a nodular mass 2 cm. above the pectinate line in the rectum. The mass was produced by a thickening of the wall of the gut but the mucosa appeared intact over the area. Microscopically, the wall was found to be almost completely destroyed by a necrotic process which had begun to include the mucosa as well. Numerous inflammatory cells were seen at the border of this lesion. Many bacteria were seen but no acid fast organisms were demonstrated.

*Lymph nodes* The lymph glands throughout the body were enlarged and firm. The histologic study revealed a complete destruction of the normal architecture. The capsule was thickened and the stroma increased. The sinusoids were dilated and contained cells. Extramedullary hemopoiesis was seen and a few megakaryocytes were present. Lymphocytes in various stages of development were noted.

*Bone marrow* Specimens were taken from the sternum, ribs, and vertebrae and all had a dry appearance. The histologic changes included complete alteration of the normal architecture. The marrow was hyperemic and there was a great increase in the number of megakaryocytes. Eosinophilic debris and young fibrous tissue were replacing the normal marrow elements and isolated islands or pockets of myeloid activity were seen. One of the rib sections showed an area of necrosis and increase in the number of small lymphocytes.

*Bacteriologic examination* All the sections were stained by the Ziehl-Neelson technic and the caseous foci found filled with acid fast organisms.

*Pathologic diagnosis* Caseous tuberculoma of the mediastinum, miliary tuberculosis involving the liver, spleen and bone marrow, extramedullary hemopoiesis in the spleen and lymph nodes, fibrosis of the bone marrow, pleura, liver, spleen and lymph nodes, phlegmonous proctitis.

# MYELOFIBROSIS ASSOCIATED WITH TUBERCULOSIS

## CASE 3

W. D., a 29 year old male, entered the hospital on January 2, 1946, complaining of weakness, backache, intermittent chills and fever with associated nausea and vomiting.

*Family history* Irrelevant

*Past history* Between 1936 and 1937 the patient was employed by General Electric X-ray Corporation and was exposed to considerable x ray radiation and phenol. His leukocyte count at that time was 6,750. In December, 1943, he had a soft mass 6 cm in diameter in the lower left side of the neck with two lymph nodes palpable below this. Aspiration of the mass was unsuccessful but it disappeared after five x ray treatments with a total of 750 r. He served a tour of military duty in the United States without illness and was discharged in March, 1945.

*Present illness* His weakness, backache, and a temperature of 99 F were first noticed in April, nine months before admission. In July, the patient had chills and fever rising to 103 F which lasted for about an hour and recurred every four to eight hours for eight days. He remained symptom free for three weeks when he had a similar attack also lasting eight days. In early October, he had a third bout. For three months he remained free from chills and fever but was weak. On December 31 he had still another attack. On each occasion nausea and vomiting appeared at the height of the febrile episodes. During this entire period, the patient had a weight loss of 25 pounds.

*Physical examination* The temperature was 100.4 F, pulse, 90 per minute, blood pressure, 110/70, and weight 128½ pounds. The heart and lungs were normal. The lymph glands and spleen were not palpable. The liver was felt one fingerbreadth below the right costal margin.

*Laboratory examination* Previous to entry, studies included tuberculin and brucella skin tests, agglutination studies for the enteric pathogens and brucella, the Davidsohn heterophile agglutination test, x ray of the lumbosacral spine, retrograde and intravenous urograms, and fluoroscopic examination of the chest and gastro intestinal tract. All of these were negative with the exception of a small gastric ulcer demonstrated by fluoroscopy. Proctoscopic and cystoscopic examinations were negative. During hospitalization, the urine had a specific gravity of 1.010, albumin one plus, sugar negative, 20-40 erythrocytes and occasional leukocytes per high power field. Direct and bacterial examinations of the stools were negative. A guinea pig inoculated with urine did not reveal any evidence of tuberculosis. The Wassermann and Kahn tests were negative. The chest x-rays repeatedly showed a few clean-cut calcified deposits on the left side radiating from the lung root outward. The last film was made about two months before death. The tuberculin skin test was again negative and the electrocardiogram was normal. The prothrombin time was 56.4 per cent of normal, the bromsulphalein showed 12 per cent retention of dye, total protein 6.28 to 3.88 Gm, albumin 3.94 to 2.15 Gm, globulin 2.34 to 1.73 Gm, icterus index 5 units and blood urea nitrogen 12.7 mg. The peripheral blood studies (table 2) showed a slowly progressive normocytic, normochromic anemia, and leukopenia. A few normoblasts and immature leukocytes were seen. The reticulocyte count was 1.8 per cent and the platelets were normal. Aspirated sternal marrow (table 3) showed hyperplasia at first, followed later by a distinct hypoplasia with an increase in megakaryocytes. No malarial parasites were found. Biopsy studies of the sternal bone marrow (fig. 2) in July revealed complete alteration of the normal architecture. The myeloid and erythroid elements were greatly reduced and widely scattered. No fatty tissue and eosinophilic debris. Biopsy material from a retroperitoneal mass at the same time was composed of large epithelioid cells with pale vesicular cytoplasm. Scattered throughout these cells was a fine network of fibrous connective tissue with new-growing fibroblasts and diffuse, small, round-cell infiltrations more dense in some areas than others. A few polymorphonuclear cells and an occasional large multi-nucleated cell with characteristics of a Dorothy Reed cell were seen. The diagnosis was an inflammatory process with many characteristics of Hodgkin's disease.

*Clinical course* The patient was discharged January 13 on his fourth afebrile day, only to be readmitted to the hospital January 23 with a recurrence of the backache and weakness followed by chills, fever, nausea and vomiting. Fine, moist, inspiratory rales were heard throughout the chest and a soft systolic murmur was audible over the tricuspid area. The liver was four fingerbreadths below the right costal margin and the spleen could be palpated subcostally. There was no adenopathy. His fever subsided March 7 and he was discharged ten days later. On March 26 the patient was readmitted with another attack.

but this time with a severe cough and pain and tenderness in the right flank. The liver and spleen were palpable as before and there was some question of a palpable mass in the region of the right kidney. The fever reached 103 F on the second day but subsided rapidly and the patient was discharged April 1. Twenty days later the patient made his last entry into the hospital. He weighed 123 pounds. The liver was palpable four fingerbreadths below the right costal margin and was tender. The spleen was again palpable. During this admission, biopsies were taken from the sternal marrow (*see Laboratory Examinations*). On July 3 a laparotomy was done and enlarged retroperitoneal tumor masses grossly resembling Hodgkin's disease or lymphosarcoma were seen. Biopsies were taken of these and also of the liver (*Laboratory Examinations*). Eight x-ray treatments were given over the abdominal mass with no improvement. His temperature ran a very septic course, going to 104 F, and showed daily and almost hourly fluctuations. He continued to lose weight and became progressively worse and on August 19 died. Before death, his peripheral blood picture revealed marked pancytopenia. His spleen and liver were



FIG 1 Liver from W D showing large areas of fibrosis

in size and edema became pronounced. Treatment throughout his illness included sulfonamides, penicillin, oral streptomycin (for seven days), atabrine, quinine, plasmoquin, emetine, salicylates, the x-ray treatment mentioned above, and four blood transfusions. None of these had any effect on the course of the disease.

#### *Necropsy*

*Thoracic cavity* Both thoracic cavities contained about 1200 cc of a clear yellow fluid.

*Lungs* The lungs were adherent to the parietal pleura by a few firm fibrous adhesions and their surfaces were covered with greyish-white raised nodules ranging from pin-head size to 0.2 cm in diameter. The cut surfaces were wet and the same nodules were seen. The microscopic appearance of the lung was that of a multitude of tiny anemic infarcts in all the sections. The centers of these foci were caseous but there was almost a complete absence of the peripheral cellular reaction so common in tuberculosis. No Langhans giant cells were observed. Many of the surrounding alveoli contained cellular debris and fibrin resembling the consolidation of pneumonia. The pleura was thickened.

*Abdominal cavity* The abdominal cavity was filled with clear yellowish fluid. There was a large, nodular, perivertebral, retroperitoneal mass in the epigastric region.

*Liver* The liver was enlarged (1650 Gm). The surface was speckled with pin-head sized, subcapsular, greyish-white nodules, a few larger yellowish nodules and firm white irregular patches.

1) The cut surface was similar except that the firm white areas were very tough and extended deeply into the parenchyma mainly about the larger vessels. Histologic examination revealed a thickened capsule and distention of the sinuses with blood. Scattered throughout the parenchyma were a multitude of tiny tubercles. Many of these contained one or two typical Langhan giant cells. A few were surrounded by the typical cellular reaction seen in tuberculosis. The large white plaques described at autopsy were composed of organized fibrous and hyalinized connective tissue and at the borders the connective tissue extended into the parenchyma, leaving islands of liver cells behind. Foci of lymphocytes were scattered throughout this fibrous tissue. An occasional megakaryocyte and a few small foci of extramedullary hemopoiesis were seen.

*Spleen* The spleen was enlarged (700 Gm) and a small accessory spleen was found. Numerous small greyish-white nodules were seen beneath the capsule and on the cut surface. Firm, white, irregular plaques on the cut surface were similar to those seen in the liver. The sections showed a thickened fibrous capsule. The sinusoids were distended with blood. The lymph follicles were completely dispersed and only an occasional small aggregate of lymphocytes could be found about a vessel. Large areas of fibrosis were seen but the most unusual finding was the widespread seeding of small tubercles as seen in the liver. There was little surrounding cellular reaction though occasional Langhan giant cells were found in the tubercles. Immature myeloid and erythroid cells and a moderate number of large cells with large, irregular, dense nuclei resembling megakaryocytes could be seen diffusely scattered throughout the sections.

*Pancreas* (fig. 3) Microscopic examination revealed an increased amount of connective tissue and several atypical tubercles similar to those previously described.

*Adrenals* The microscopic section showed an increase in fibrous tissue and numerous small atypical tubercles.

*Retroperitoneal lymph nodes* The retroperitoneal mass was an irregular, enlarged, adherent group of lymph nodes which when sectioned showed occasional small yellowish foci. Histologic study showed a complete obliteration of the normal architecture with a greatly increased stroma. Much chronic granulomatous tissue was present with a few scattered lymphocytes, an occasional multinuclear Sternberg-Reed type of cell and atypical tubercles. Nerve bundles were seen encased in the fibrous tissue.

*Bone marrow* (fig. 4) Bone marrow taken from the sternum, ribs and vertebrae showed almost complete obliteration of the marrow cavities by dense fibrous tissue. Only a very few normal myeloid and erythroid foci could be seen. Megakaryocytes were prominent and an occasional atypical tubercle containing acid-fast organisms was found.

*Bacteriologic examination* All the sections were stained by the Ziehl-Neelson technic, the caseous foci everywhere and the connective tissue of the liver, spleen and lymph nodes were filled with acid fast organisms. These acid-fast organisms were cultured on glycerin-egg media, inoculated into a series of laboratory animals and tested as to streptomycin sensitivity. The organism was proven to be a human type of *M. tuberculosis*, possibly of low virulence and sensitive to streptomycin.

*Pathologic diagnosis* Diffuse fibrosis of the liver, spleen, lymph nodes, adrenals and bone marrow, with interacinar fibrosis of the pancreas, extramedullary hemopoiesis in the spleen and liver, generalized miliary tuberculosis.

#### CASE 4

M. B., a 36 year old housewife entered the hospital June 23, 1945, complaining of weakness, night sweats, headache, chest pain and a nonproductive cough.

*Family history* A sister died from tuberculosis.

*Past history* Irrelevant.

*Present illness* The patient was apparently well and healthy until after the normal delivery of a baby October 11, 1944. Two days postpartum an enlarged spleen was found and subsequently a diagnosis of Banti's disease was made. A splenectomy was done November 14, 1944. The pathologic report on the spleen suggested Hodgkin's disease. Following operation the patient gained 35 pounds and resumed her normal duties. In May 1945, her menstrual period lasted twelve days and she passed numerous large clots. At this time she caught cold and her temperature rose to 103 F for a few days. She continued

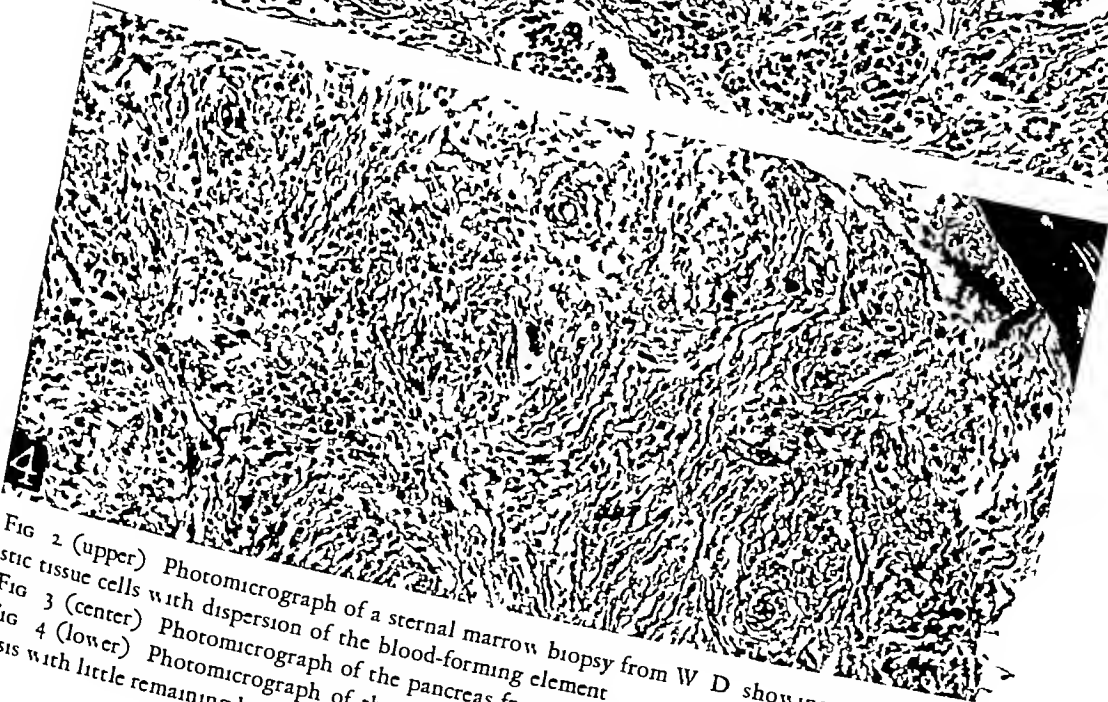
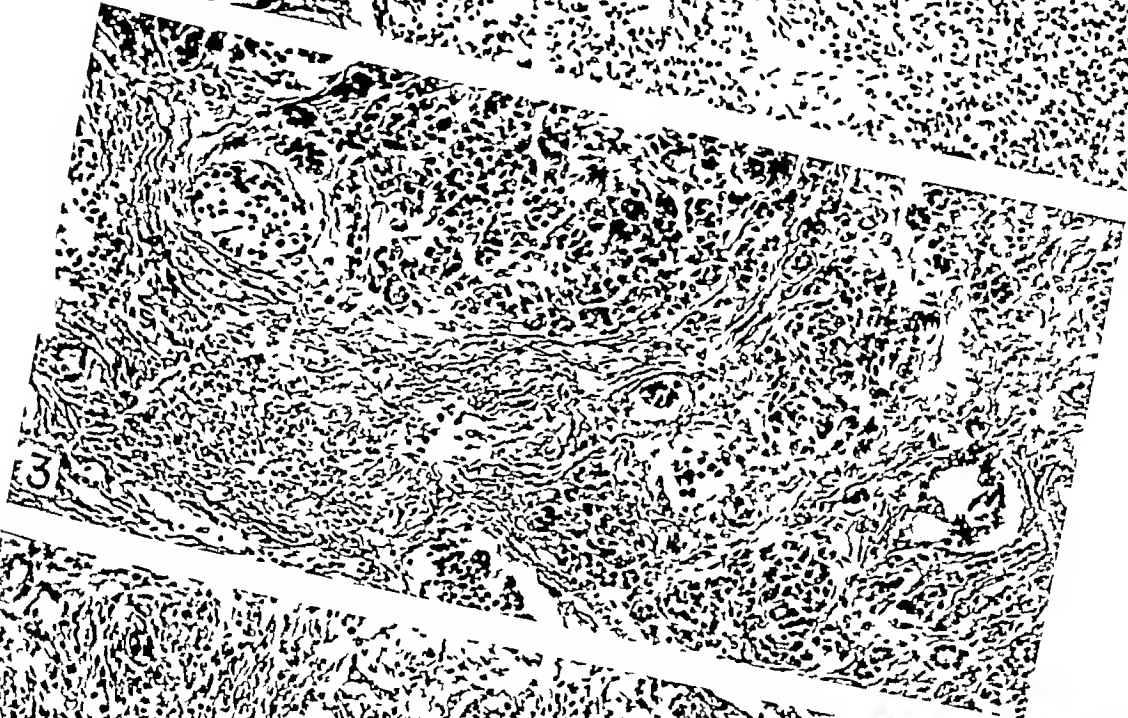
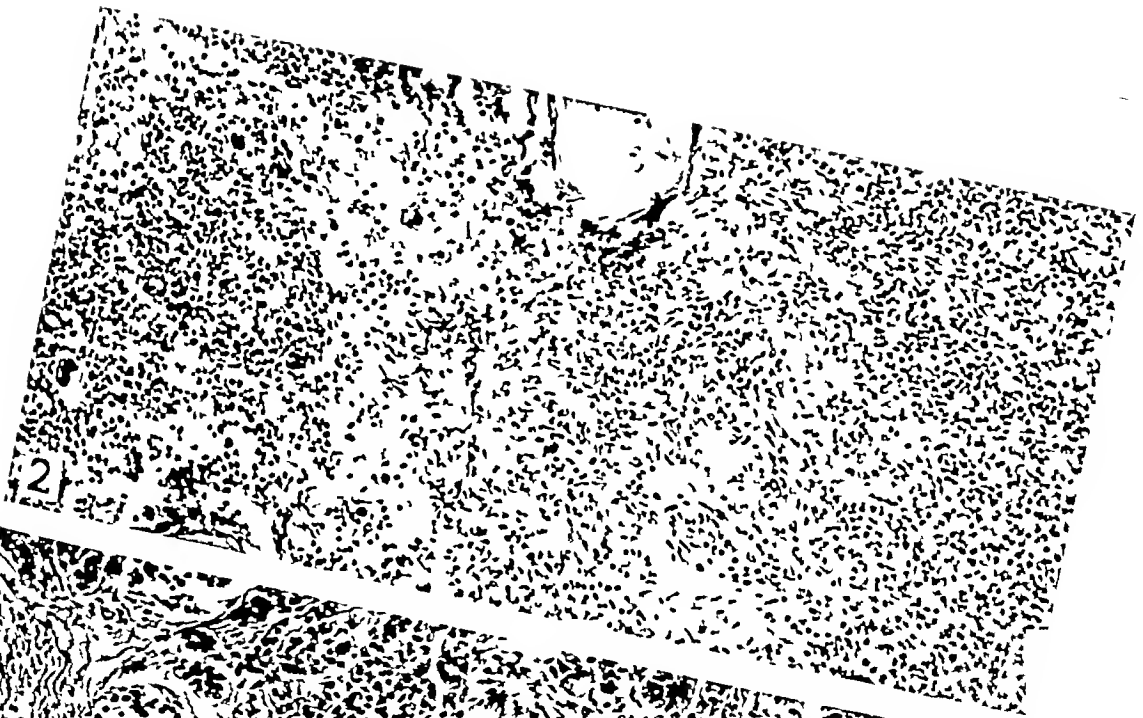


FIG 2 (upper) Photomicrograph of a sternal marrow biopsy from W D showing infiltrating fibroblastic tissue cells with dispersion of the blood-forming element  
 FIG 3 (center) Photomicrograph of the pancreas from W D showing interacinar fibrosis  
 FIG 4 (lower) Photomicrograph of the bone marrow from W D at autopsy showing extensive fibrosis with little remaining hemopoietic tissue

to feel ill and began to lose weight. Weakness then became her major complaint and was soon followed by night sweats, a persistent headache, pain in the chest and back and an irritating, nonproductive cough.

*Physical examination* Her temperature was 100.2 F, pulse, 100 per minute, blood pressure, 130/72, and respirations, 24 per minute. A soft systolic murmur was audible over the base of the heart. The lungs were normal. The liver was enlarged, extending approximately five fingerbreadths below the right costal margin. A moderate generalized lymphadenopathy was noted.

*Laboratory examination* The urine contained a trace of albumin and 7-10 leukocytes per high power field. The Kahn test was negative and blood chemistry studies, including the icterus index, were all normal. The BMR was plus 20 per cent and the electrocardiogram was normal. X-ray examinations of the chest were repeatedly normal until a few days before death when a diffuse flocculant increase in density was seen involving both lung fields. This was most pronounced at the bases and suggested an acute pulmonary edema. The peripheral blood studies (table 2) showed a predominance of young myeloid cells with a great increase in the number of megakaryocytes. The pathologic sections from the spleen were re-examined and reported suggestive of myelogenous leukemia.

*Clinical course* The treatment consisted of repeated blood transfusions. The patient grew progressively weaker and continued to lose weight. Her temperature was high, the peaks ranging between 104 F and 105 F and there was delirium. In the last week of her life, ascites and jaundice were present. She died September 21, 1945.

#### *Necropsy*

*Thoracic cavity* Both pleural cavities were obliterated by firm adhesions between the visceral and parietal pleurae.

*Lungs* The pleural and cut surfaces of the lungs were studded with numerous small greyish-white, firm nodules varying in size from 0.1 to 0.5 cm. On microscopic study, tiny areas of focal necrosis were surrounded by a zone of immature blood cells and phagocytes. Foci of extramedullary hemopoiesis and numerous megakaryocytes were seen.

*Abdominal cavity* Five hundred centimeters of dark, straw-colored fluid were found in the abdominal cavity. The visceral and parietal peritoneum was studded with small greyish-white nodules.

*Liver* The liver was enlarged (2670 Gm). The capsule and cut surfaces were covered with small tubercles. Histologic study (fig. 5) showed areas of focal necrosis and infarction surrounded by zones of hemopoiesis. The liver cords were small and surrounded by hyalinized connective tissue which obliterated many of the sinuses. Foci of extramedullary hemopoiesis and megakaryocytes were seen.

*Pancreas* Microscopically, there was a pronounced periductile, perivascular and periglandular fibrosis.

*Adrenals* The sections showed fibrous tissue replacement with separation of the cortical and medullary cells and microscopic areas of caseation and foci of extramedullary hemopoiesis.

*Bone marrow* The cortices of the ribs, sternum, and vertebrae were thickened and the trabeculae prominent. The medullary spaces were filled with a dry, fibrous-like tissue. Microscopically, the normal marrow was completely distorted (fig. 6). Relatively few hemopoietic cells remained and these were widely dispersed through the connective tissue filling the marrow spaces. The megakaryocytes appeared increased in number. Necrotic foci were also found.

*Lymph nodes* The mediastinal, retroperitoneal and perivertebral lymph nodes were enlarged and matted together. Microscopic study of these lymph nodes showed proliferating connective tissue infiltrated with immature blood cells and a few megakaryocytes. Many of the nodes contained focal areas of necrosis.

*Bacteriologic examination* All of the sections were stained by the Ziehl-Neelsen technique and huge numbers of acid fast organisms were found in the areas of necrosis in all the organs.

*Pathologic diagnosis* Extramedullary hemopoiesis in the liver, lung, spleen and lymph nodes, extensive fibrosis of the liver, spleen, pancreas, adrenals, lymph nodes and bone marrow, generalized acute, diffuse, miliary tuberculosis.

#### ANALYSIS OF CASES

A summary of the clinical, hematologic and pathologic data of the four cases of myelofibrosis with tuberculosis are recorded in tables 2, 3 and 4. We studied W. M., W. D. and M. B. during life and the records of C. B. several years after death. M.



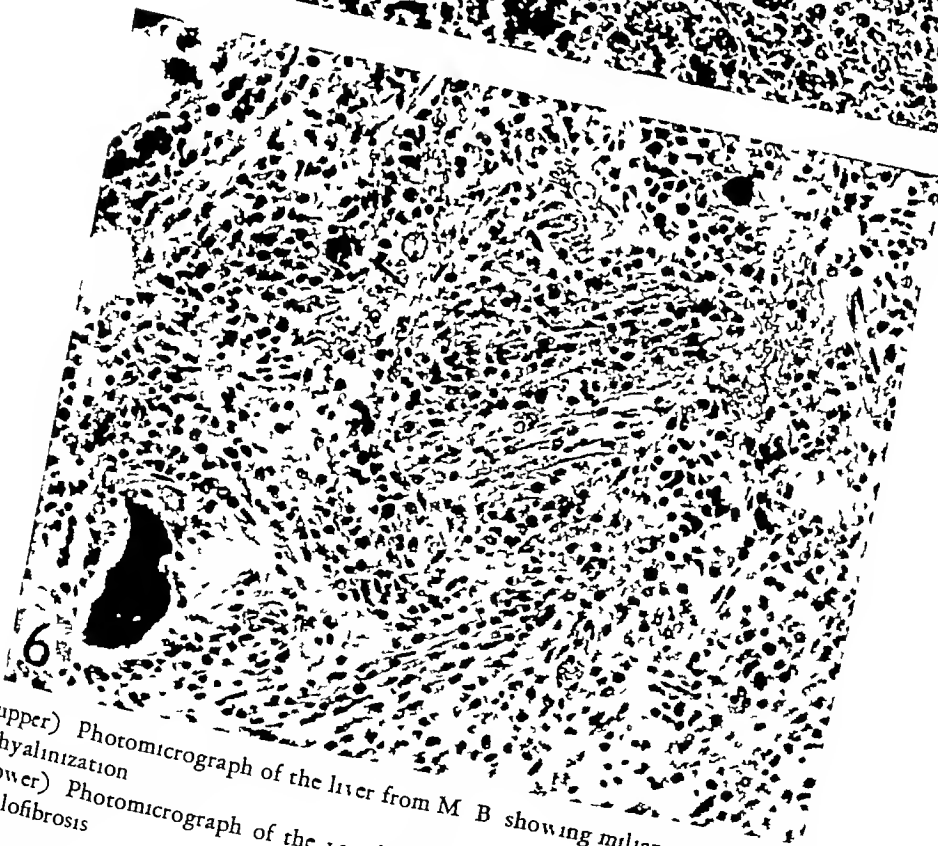
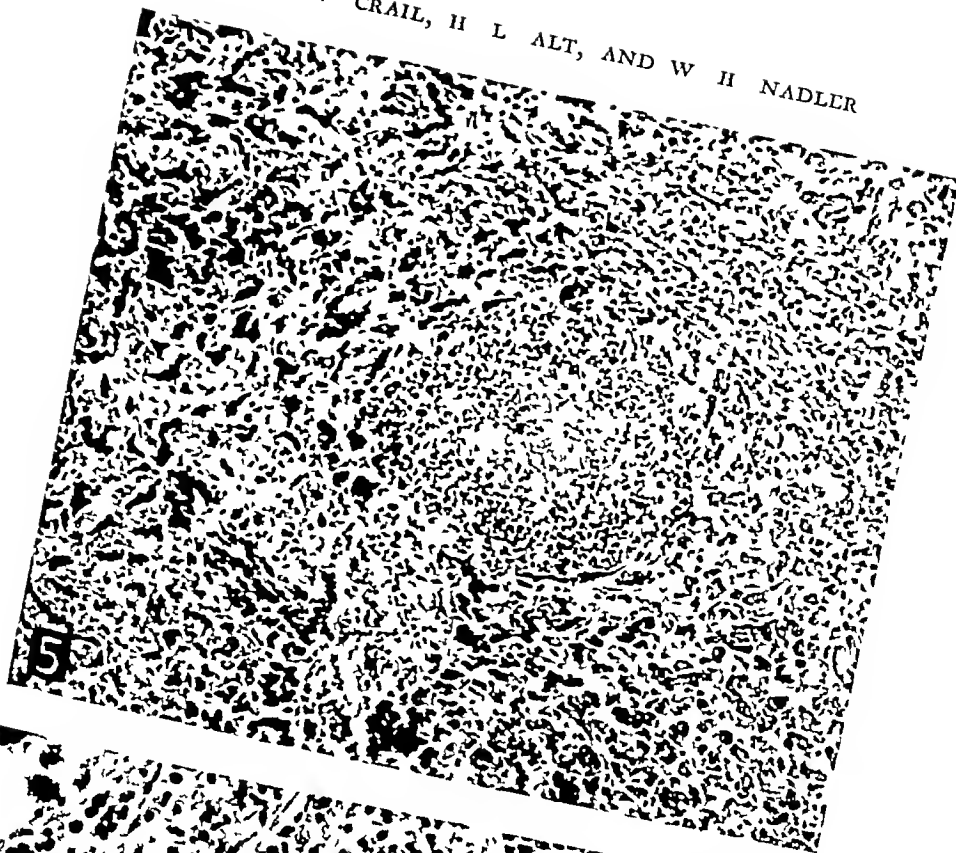


Fig 5 (upper) Photomicrograph of the liver from M B showing miliary tubercle with associated fibrosis and hyalinization  
 Fig 6 (lower) Photomicrograph of the vertebral bone marrow from M B at autopsy showing extensive myelofibrosis

B has been reported<sup>12</sup> previously but is included to make the present study more complete. The diagnosis of tuberculosis was made antemortem in C B and was considered but not established in W D.

*History* The primary complaints of these cases were pain in the long bones, back



or abdomen, fatigue, progressive weakness, pallor and loss of weight. Their *ages* ranged between 29 and 50, averaging  $33\frac{1}{2}$  years. The *sex distribution* was equal. A definite *history of a tuberculous* contact was obtained in M. B. W. D. gave a history of cervical adenopathy which was suggestive of tuberculosis.

*Physical findings.* All of the patients showed a daily afternoon *fever* with occasional spikes to 104 F. During the last few months, the daily elevations frequently went above this mark. W. D. had repeated recurrences of chills and fever, sometimes two or three a day with afebrile remissions lasting eight to twenty days. The *spleen* in each case was moderately enlarged. Splenectomies were done on C. B. and M. B. early in their illnesses with probable adverse effects. The *livers* were markedly enlarged, smooth and nontender in three cases. In C. B., the liver was palpable only on deep inspiration. Generalized *lymphadenopathy* was moderate in all cases. The retroperitoneal lymph nodes were greatly enlarged in W. D. and were palpable through the abdominal wall as a firm, epigastric mass.

*Laboratory findings.* A refractory *anemia* was present in all cases. The anemia was profound throughout the illness of W. M. and he required frequent transfusions. In the remaining cases, the anemia was slowly progressive but became terminally pronounced. A *leukemoid reaction* characterized by the presence of immature leukocytes and normoblasts occurred in each case. Three cases (C. B., W. M. and W. D.) had a progressive *leukopenia*. On the other hand, M. B. had a leukocyte count above 100,000 and the differential closely resembled an acute myelocytic leukemia. The *platelet* count paralleled the leukocyte count, it was reduced to the lower limits of normal in three cases and was well above 500,000 in M. B. Giant platelets and megakaryocytes were seen in peripheral blood smears of the latter case. In each case, the *reticulocyte count* was normal. The *sternal marrow* aspirations in W. M. and W. D. early revealed a hyperplastic marrow. Later the marrow became hypoplastic and sternal aspirations then resembled the peripheral blood both in total and differential cell counts. The number of megakaryocytes was increased in each case. Single marrow aspirations on the two remaining cases showed hypoplasia with an increase in the megakaryocytes. A bone marrow biopsy was done on only one patient (W. D.). The sections revealed a depletion of the normal hemopoietic tissue and fat with beginning fibroblastic replacement and a marked increase in the megakaryocytes. One patient (W. D.) consistently had a low grade hematuria. *Stool* examinations for blood were repeatedly negative in all instances. Uniform *blood chemistry* studies were not carried out on these patients, however, W. D. and M. B. showed evidence of a slightly decreased liver function. The icterus index was normal in each case. *Bacteriologic* and *serologic studies* were repeatedly negative in all but C. B., where acid-fast organisms were recovered from fluid aspirated from the chest late in the illness. Unfortunately, tuberculin skin tests were made in only one case (W. D.) and were reported negative. *Roentgenologic examinations* of the chest revealed evidence of old, healed tuberculosis in each case. Terminally, pleural effusion was found in C. B. and mottling suggestive of edema in M. B. Since miliary tubercles were found in the lungs at autopsy, serial roentgenograms might have been of diagnostic aid late in the disease.

*Clinical course.* All patients ran a continued downhill course with progressive

weakness, loss of weight and fever ending in death C B and M B developed terminal jaundice The *duration of illness* ranged from twelve to eighteen months with an average of 16.2 months

*Necropsy findings* Generalized, caseating, *miliary tuberculosis* was found in all of the organs in these cases These lesions were small but were found filled with large numbers of acid-fast organisms *Extramedullary hemopoiesis* and an increase in *megakaryocytes* were evident in every instance, most commonly in the spleen, lymph nodes and liver The *lymph nodes* in each case were enlarged and the normal cellular elements were replaced by proliferating granulomatous tissue *Bone marrow* from the sternum, rib and vertebra in each case was replaced by varying amounts of connective tissue which was confirmed by special staining \* The most massive fibrosis was present in W D where it had progressed extensively since the time of biopsy three months before death (figs 2 and 4) *Fibrosis* and *hyalinization* of organs other than the bone marrow, particularly of the liver, (figs 1 and 5) spleen, pleura, pancreas (fig 3) and adrenals were prominent in all of our cases

#### *Comparison of the Four Tuberculous Cases with Five Cases of Idiopathic Myelofibrosis*

We have observed and analyzed 5 cases of idiopathic myelofibrosis (table 4) which will be reported elsewhere Three are still living and 2 have died These patients have many features in common with the tuberculous group, namely the splenomegaly, anemia, leukemoid blood picture, fibrosis in the bone marrow with an increase in megakaryocytes and extramedullary hemopoiesis On the other hand, there were certain differences in the two groups The idiopathic cases were in an older age group (53 to 77 years), there was no fever except terminally in one case, the spleens were larger and lymphadenopathy was less prominent The average duration of illness in this group at the time of writing was thirty-two and one-half months as compared to sixteen months in the tuberculous patients There was no evidence of active tuberculosis in the idiopathic group In general, the idiopathic cases resembled the tuberculous cases hematologically, but the latter group ran a septic course and terminated fatally within a shorter time

#### DISCUSSION

*Pathogenesis* The possible role of tuberculosis in the production of myelofibrosis has been considered by previous writers (*see* Review of the Literature) However, there is no clean-cut evidence in favor of this relationship In a preliminary study of this subject, one of us (H W C)<sup>12</sup> proposed that the acid-fast organisms found in the atypical tubercles of M B and in other cases reported in the literature may be responsible for the myelofibrosis Furthermore, since the atypical tubercles in these cases resembled the lesions produced experimentally with avian tuberculosis, it was suggested that the organisms be identified in subsequent investigations This was done in W D where the acid-fast bacilli were obtained at autopsy and identified according to the method described by Feldman<sup>13</sup> The organism in this case was found to be a human tubercle bacillus, possibly of low virulence and definitely sensitive to streptomycin

\* Mallory's connective tissue stain



Acute caseating miliary tuberculosis is the name Rich<sup>14</sup> applied to the atypical disease seen in our cases. He points out this disease may be overlooked at autopsy or, if recognized, may be misinterpreted as avian tuberculosis. Rich<sup>14</sup> has experimentally produced the lesions of caseating tuberculosis by injection of large numbers of bacilli into the blood stream of a hypersensitive animal. They become widely disseminated and are characterized by tiny caseous foci without epithelioid cells, lymphocytes or giant cells, the organisms multiply rapidly in these lesions and large numbers are found at autopsy. If the animals survive, the organisms are reduced and typical tubercles develop. Complete healing may take place with or without scar formation. Pinner<sup>15</sup> believes that this type of reaction is due to an atypical response of the host. Clinically and experimentally, this massive dissemination of organisms is characterized by toxemia and spiking temperature elevations.

The proliferative reactions in tuberculosis have led to widespread investigations. According to Rich,<sup>14</sup> the factors influencing fibrosis are as follows: (1) virulence of the organism, (2) race, (3) presence and degree of immunity or hypersensitivity, and (4) resistance of the host. Sabin<sup>16</sup> studied the tissue response to various fractions of acid-fast organisms. She found that an unsaponified higher alcohol derived from the waxes produced a remarkable proliferation of fibroblasts both diffusely and in small clumps. Kaufmann<sup>17</sup> described a relatively benign form of tuberculosis involving the lymph nodes which he called 'attenuated tuberculosis'. It runs a chronic course with massive enlargement of the glands due to a proliferative reaction. There is little or no caseation. The organisms are often difficult to demonstrate. Pinner<sup>15</sup> describes sarcoidosis as a hematogenous tuberculosis with a productive tissue response and no caseation. He believes this is an expression of a high degree of specific resistance as manifested by the benign course, the absence of tissue destruction and toxic symptoms and the efficient destruction of tubercle bacilli shortly after focalization has taken place. Such a concept embraces a large field of fibrotic and hyalinized lesions resembling tuberculosis. Ewing<sup>18</sup> and L'Esperance<sup>19</sup> have strongly supported the concept of a tuberculous etiology of Hodgkin's disease. Arneith,<sup>20</sup> Muller,<sup>21</sup> Pinner<sup>15</sup> and others<sup>22</sup> occasionally observed hematologic findings in miliary tuberculosis similar to those described in our cases. These authors point out that a shift to the left of the granulocytes is one of the heralding features of active, progressive tuberculosis. This may be accompanied by a leukocytosis or a leukopenia. \* Leukemoid reactions often accompanied by a leukocytosis and the presence of myeloblasts do occur and are at times difficult to differentiate from leukemia.<sup>23</sup> A low grade or moderate anemia is common in pulmonary tuberculosis. However, Pinner<sup>15</sup> states that marked degrees of anemia (below 75 per cent) are strongly indicative of extrapulmonary involvement or of nontuberculous complications. Marrow studies in miliary tuberculosis have shown early hyperplasia of all the hemopoietic tissue and later hypoplasia or aplasia. Muller,<sup>21</sup> in some of his cases, describes marrow of generalized tuberculosis, filled with eosinophilic debris.

\* Recently, in our clinic, autopsy has been performed on a patient who had miliary tuberculosis associated with a hypoplastic marrow without fibrosis. During life, she had a leukoerythroblastic anemia.

and sometimes connective tissue fibrils with islands of hyperplastic myelopoietic tissue. Further evidence that tuberculosis depresses the marrow is demonstrated by the suppression of leukemia by an active tuberculous infection (Jaffe,<sup>24</sup> Heinle and Weir<sup>25</sup> and Ulrich and Parks<sup>26</sup>)

Undoubtedly the atypical miliary tuberculosis seen at autopsy in the cases reported here and in the literature represent a terminal dissemination of the type described by Rich.<sup>14</sup> However, a review of the clinical course, physical findings, hematologic and pathologic examinations coupled with the autopsy findings and bacteriologic studies, strongly suggest a protracted, progressive granulomatous disease of tuberculous origin. The degree and extent of fibrosis of the marrow and other organs may be dependent upon factors previously enumerated and upon the length of life of the patient. The marrow fibrosis in these cases is considered to be part of a generalized disease.

**Diagnosis.** The possible diagnosis of myelofibrosis must be entertained when a patient complaining of bizarre pain, weakness and weight loss is found to have splenomegaly, hepatomegaly, lymphadenopathy and hematologic findings of refractory anemia with a leukemoid reaction. Tuberculous involvement is to be thought of if there is in addition a recurrent, spiking or persistent unexplained fever.

A sternal aspiration in myelofibrosis reveals a hypocellular marrow with an increase in the megakaryocytes. The tuberculous case frequently shows an initial marrow hyperplasia followed by hypoplasia. This material must be carefully studied for tuberculosis as described by Schleicher.<sup>27</sup> A *bone marrow biopsy* must be obtained to confirm the diagnosis of myelofibrosis. Lymph node biopsies and splenic punctures may be of assistance. These tissues should be cultured, inoculated into guinea pigs and examined pathologically for acid-fast organisms. X-ray examination of the chest may reveal evidence of miliary tuberculosis but the absence of findings does not rule out this disease. With the proper diagnostic approach it should be possible to make the diagnosis of myelofibrosis with tuberculosis antemortem in many cases.

**Treatment.** Blood transfusions temporarily raise the oxygen carrying power of the blood but have no effect on the course of the disease. As is to be expected, there is no response of the anemia to liver and iron, and sulfonamides and penicillin have no effect on the fever. Splenectomy and irradiation are contraindicated as they reduce the compensatory hemopoietic mechanism and probably hasten death. Streptomycin offers the only hope in the treatment of this disease. Recent reports indicate that certain cases of generalized tuberculosis<sup>28</sup> and tuberculous meningitis<sup>29</sup> are benefited by adequate treatment with streptomycin. When the diagnosis is made, it would seem logical to give 2 to 3 Gm. of streptomycin daily over a prolonged period of time. Since the disease is characterized by spontaneous remissions, one must be guarded in interpreting therapeutic results.

#### SUMMARY

Myelofibrosis (fibrotic bone marrow and, usually, an increase in megakaryocytes) is characterized by generalized pains, weakness, loss of weight, enlargement of the liver and spleen and a leuko-erythroblastic anemia.

Four cases of myelofibrosis associated with generalized tuberculosis have been reviewed in detail. Autopsy examination of the 4 cases revealed acute, caseating tuberculosis which was considered to be responsible for the bone marrow and generalized fibrosis observed. A similar type of tuberculosis occurred in 7 of 91 cases of myelofibrosis reviewed in the literature. The pathogenesis of myelofibrosis associated with tuberculosis is discussed.

In the diagnosis of this syndrome, attention is called to the importance of obtaining a bone marrow biopsy and making a complete bacteriologic and pathologic study of this tissue for tuberculosis.

The 4 tuberculous cases here reported as compared with 5 cases of idiopathic type, are younger, have hyperpyrexia, less splenic but greater lymph node enlargement and run a shorter course before death.

#### ACKNOWLEDGMENT

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# THE OCCURRENCE OF NORMOBLASTS IN THE PERIPHERAL BLOOD IN CONGESTIVE HEART FAILURE AN INDICATION OF UNFAVORABLE PROGNOSIS

By J GROEN, M D , AND E G GODFRIED, M D

**D**URING THE PAST year the authors have observed 9 patients with severe congestive heart failure, all of whom showed a varying number of normoblasts in the peripheral blood. In some of these cases there was a temporary remission of the sequellae of the heart failure, during this remission the normoblasts disappeared from the peripheral blood. However, in all cases the patients died.

## CASE REPORTS

*Case 1* C D , a 34 year old woman, was admitted on June 12, 1946, because of decompensated mitral stenosis. She was slightly jaundiced, the liver was greatly enlarged, there were infarcts in both lungs. Five per cent normoblasts were found in the peripheral blood. The patient died on July 2, 1946. The diagnosis was confirmed at autopsy.

*Case 2* S W , a 56 year old woman, entered the hospital on May 9, 1946, because of dyspnea, from which she had been suffering during the previous six months. She was cyanotic, dyspneic, and had slight jaundice. On examination, a mitral insufficiency, stenosis and auricular fibrillation were found. The liver was much enlarged, there was extensive edema. During this period, 10 and 17 per cent, respectively, of normoblasts were found on two occasions in the peripheral blood, but these disappeared within three days when her condition improved. In spite of this remission, the patient died on May 22, 1946. On the day before her death she had another infarction of the lung.

On May 13, a sternal puncture had been performed. The bone marrow was found to be essentially normal, but the presence of a relatively great number of normoblasts and erythroblasts was noted (about 25 per cent of all nucleated cells).

The diagnosis of valvular heart disease was confirmed at autopsy, there were many infarcts in both lungs and a thrombosis of the left auricle of the heart.

*Case 3* A G , a 20 year old woman, entered the hospital on May 4, 1946, with cardiac decompensation, due to insufficiency and stenosis of the mitral valve. She was dyspneic but not cyanotic. There was no jaundice. The liver was enlarged. There was massive edema of the legs. The blood count was as follows:

	May 6, 1946	May 7, 1946
Leukocytes	12,800	17,000
Erythrocytes	3,640,000	3,900,000
Hemoglobin	61	63
Normoblasts	—	7
Myelocytes	1	0.5
Metamyelocytes	0.5	0.5
Stabs	5.5	9
Polynuclear cells	54.5	69
Eosinophiles	0.5	—
Monocytes	2.5	2.5
Lymphocytes	35.5	18.5

There was a marked anisocytosis, poikilocytosis, polychromasia, anisochromia and macrocytosis. The patient died on May 8, 1946. The autopsy showed a verrucous endocarditis (of the mitral and aortic valves), thrombi in the left auricle of the heart, congestion, fatty degeneration and regeneration of the liver.

*Case 4* A B , a 34 year old man, was admitted on June 28, 1946, with a diagnosis of insufficiency and

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stenosis of the mitral valve and aortic stenosis. He was cyanotic and extremely dyspneic. There was slight jaundice. The liver was palpable. There was no edema. The patient had distinct anginal pain. An electrocardiogram showed changes that were typical of a severe myocardial lesion. The blood picture was as follows: leukocytes, 7,000, erythrocytes, 5,000,000, hemoglobin, 100, normoblasts, 3, stabs, 4, polynuclear cells, 80, monocytes, 1, lymphocytes, 15.

The patient died on July 5, 1946. No autopsy was performed.

*Case 5* H S, a 71 year old man, was admitted on March 4, 1946, with cardiac asthma. He had been known for years to have suffered from hypertension. The patient was extremely cyanotic. There was slight jaundice and very severe dyspnea. He showed all the signs of congestive heart failure. There was extensive edema of the legs, ascites and hydrothorax. One normoblast was found per 100 nucleated cells. When the signs of heart failure disappeared, normoblasts were no longer seen. The patient died November 4, 1946. Autopsy showed hypertrophy of the heart, multiple emboli in both lungs and chronic glomerulonephritis.

*Case 6* P H, a 48 year old woman, was admitted on November 9, 1946, with mitral insufficiency and stenosis. She was dyspneic and cyanotic. No jaundice was present. There was gangrene of the tip of the nose and of both lower legs. No pulsations were felt in the left femoral and the right popliteal arteries. There was an infarct in the right lung. The blood count was as follows: leukocytes, 22,000, erythrocytes,

TABLE 1—*Blood Picture of S W (Case 2)*

	5/10	5/11	5/13	5/14	5/16	5/18	5/21
Leukocyte count	12,500			12,700	12,400		26,000
Erythrocyte count	5,250,000				5,360,000		5,310,000
Hemoglobin	77				80		82
Normoblasts	10	17	3				
Myelocytes			1				
Metamyelocytes	1	1					
Stabs	4	7	4	4	4	4	7
Polynuclear cells	82	81	86	88	89	79	84
Monocytes	1	2	1	4	3	1	
Lymphocytes	12	9	8	4	4	16	9

4,020,000, hemoglobin, 59, normoblasts, 1, stabs, 6, polynuclear cells, 80, eosinophiles, 1, monocytes, 3, lymphocytes, 10.

The patient died on November 16, 1946. The autopsy revealed a thrombus in the left auricle of the heart, an infarct of the lower lobe of the right lung, an embolic obturation of the left iliac artery and congestion of the liver.

*Case 7* T V, a 45 year old woman, was admitted on November 19, 1946. She had a history of chorea twenty-seven years previously. A diagnosis of mitral insufficiency and stenosis was made. The patient had auricular fibrillation and multiple infarctions of both lungs. There was gangrene of the tip of the nose and of the right foot, the latter was caused by an obturation of the right femoral artery. The patient was extremely dyspneic and deeply cyanotic. There was no jaundice. An electrocardiogram showed bundle branch block. The blood count was as follows: leukocytes, 2,800, erythrocytes, 4,500,000, hemoglobin, 78, normoblasts, 4, myelocytes, 1, metamyelocytes, 1, stabs, 1, polynuclear cells, 78, monocytes, 1, lymphocytes, 14.

The patient died on November 20, 1946. At autopsy the clinical diagnosis was confirmed: valvular heart disease, thrombosis of the right auricle, multiple infarctions in both lungs. A small amount of fluid was found in both pleural cavities. Cardiac cirrhosis of the liver with regeneration was also present.

*Case 8* T S, a 40 year old woman, was admitted for the first time on April 24, 1946, because of mitral insufficiency and stenosis, with auricular fibrillation. She had a greatly enlarged liver with ascites. The patient left the hospital on July 6, and was readmitted on August 30. On this second admission she was extremely dyspneic, cyanotic and subicteric. On October 1, a few normoblasts were found in the peripheral blood. The patient died on October 14, 1946. At autopsy, the mitral valve lesions were found,

in addition, she had a tricuspid stenosis and there was a thrombus in the right atrium. The liver was cirrhotic.

*Case 9* J. D., a 63 year old woman, was admitted on November 13, 1946, with a diagnosis of mitral insufficiency and stenosis, with auricular fibrillation, and decompensation. She was slightly dyspneic, and deeply cyanotic. There was no jaundice. Infarctions were present in both lungs. The blood count was as follows: leukocytes, 5,600, erythrocytes, 4,300,000, hemoglobin, 88, normoblasts, 2, polynuclear cells, 66, eosinophiles, 2, monocytes, 5, lymphocytes, 27.

The patient died on February 5, 1947. The clinical diagnosis was confirmed at autopsy, there were multiple infarctions in both lungs.

### DISCUSSION

The appearance of normoblasts in the blood of patients with congestive heart failure is mentioned only twice in the literature. The standard textbooks of diseases of the blood and of the heart describe the finding of normoblasts in the peripheral blood only in relation to congenital heart disease accompanied by cyanosis. In

TABLE 2a—*Cabot's cases*

Patient	Age	Clinical data	Hematologic data	Remarks
Case I	8	Mitral disease, nephritis	Hb 35, 2% normoblasts, leukocytes 220,000	Died
Case II	25	Mitral disease, aortic insufficiency	Hg 35, 1% normoblasts, leukocytes 13,000	Died
Case III	30	Mitral disease, aortic insufficiency	1st day Hb 10, 8% normoblasts, leukocytes 6,000 7th day Hb 10, 4% megaloblasts, 7% normoblasts, leukocytes 4,200 15th day Hb 15, leukocytes 10,400 20th day Hb 12.5, 1% megaloblasts, leukocytes 5,000	'

acquired valvular disease, and in other forms of congestive heart failure, this sign seems to have been overlooked by the majority of investigators.

Cabot<sup>1</sup> mentions, in the fifth edition of this book, 'The Clinical Examination of the Blood,' three cases with decompensated mitral disease in which a severe anemia was present. The blood contained nucleated red cells. All three were chronic cases without active endocarditis. He does not give further details, and there are no data regarding the presence of infarcts in the lungs. Two of Cabot's patients died, the fate of the third is not mentioned.

In 1931, Frank and Hartmann<sup>2</sup> described six patients with various types of right-sided heart failure in whom normoblasts were found in the peripheral blood. All cases showed cyanosis and some degree of jaundice, and all of them died. The cases of Frank and Hartmann were not anemic.

Table 2 summarizes the authors' observations, and some of the data on the cases described by Cabot, and Frank and Hartmann. From this table it is evident that all the cases showed a very severe insufficiency of the heart. It is also clear that it was

TABLE 2b—*Frank and Hartmann Cases*

Patient	Age	Clinical data	Hematologic data	Remarks
Case I	52	Anginal complaints, dyspnea, pleural transudate, anasarca	Hb 92, 7% erythroblasts	Autopsy Coronary sclerosis, myodegeneration of heart, aneurism of left ventricle with thrombosis
Case II	37	Insufficiency and stenosis of mitral and aortic valves, insufficiency of tricuspid valve, decompensation, enlarged liver, slight jaundice, infarctions in lungs	Hb 48, 1% macroblasts, 4% normoblasts, leukocytes 25,000	Died No autopsy
Case III	39	2 years before admission, amputation of leg for gangrene caused by endarteritis obliterans, pneumonia, slight jaundice	Hb 90, 4% erythroblasts, leukocytes 17,200 3 days later Hb 88, 14% erythroblasts, leukocytes 19,000 5 days later Hb 87, per 100 white cells 175 erythroblasts, 14 macroblasts, leukocytes 14,800	Autopsy Thrombus in left ventricle and right atrium, arteriosclerosis and formation of a thrombus in right coronary artery, myocardial infarction Thrombanguitis obliterans of abdominal aorta
Case IV	54	Coronary thrombosis, edema of legs, jaundice	Hb 67, 4% erythroblasts, leukocytes 10,000	Autopsy Coronary thrombosis, aneurism of left ventricle of the heart with a large thrombus
Case V	34	Strong cyanosis, edema	2nd day Hb 87 per 100 white cells, 64 erythroblasts, 6 macroblasts, leukocytes 21,400 3rd day Hb 87 per 100 white cells, 52 macroblasts, 25 erythroblasts, leukocytes 17,700	Autopsy Hypertrophy of right ventricle, thrombi in left ventricle, thrombosis of right pulmonary artery
Case VI	42	Insufficiency and stenosis of mitral valve, insufficiency of tricuspid valve, extreme cyanosis, extreme dyspnea, jaundice, edema	5th day Hb 78, 2% normoblasts, leukocytes 10,500 11th day 1% macroblasts, 2% erythroblasts 12th day 7% erythroblasts, 3% macroblasts 13th day 4% erythroblasts	Autopsy Chronic endocarditis of mitral and tricuspid valve, atrophy and regeneration of liver, jaundice

the decompensation and not any specific type of heart disease that caused the normoblastosis. Among the patients showing the phenomenon there were cases

TABLE 2C—*Authors observations*

Patient	Age	Clinical data	Hematologic data	Remarks
Case I	34	6/12 Stenosis and insufficiency of mitral valve, auricular fibrillation, congestion of lungs, orthopnea, cyanosis, edema of legs 6/15 Jaundice 6/17 Infarction in lung 6/30 Greatly enlarged liver 7/2 Died	6/19 5% normoblasts 6/21 3% normoblasts, Hb 59 6/25 1% normoblasts 6/27 1% normoblasts	Autopsy Chronic pancarditis, hypertrophy of heart, thrombosis in both ears of heart, infarction in lung, edema of legs
Case II	56	5/9 Insufficiency and stenosis of mitral valve, auricular fibrillation, enlarged liver, edema of legs, orthopnea, cyanosis, slight jaundice 5/13 Injection of salyrgan, followed by a large diuresis 5/22 Died	5/10 Hb 77, 10% normoblasts, leukocytes 12,500 5/11 17% normoblasts 5/13 4 hrs after injection of salyrgan 3% normoblasts From 5/14 onward, no normoblasts	5/12 Sternal puncture—bone marrow almost normal, 25% of nucleated cells are normoblasts and erythroblasts Autopsy Recurrent endocarditis of mitral valve, hypertrophy of heart, infarction in lungs, thrombosis of left heart-ear
Case III	20	5/4 Insufficiency and stenosis of mitral valve, enlarged liver, edema of leg 5/8 Died	5/6 Hb 61, no normoblasts, leukocytes 12,800 5/7 7% normoblasts, leukocytes 17,000	Autopsy Verrucous endocarditis (mitral and aortic valves), thrombi in left heart-ear, congestion of the liver with fatty degeneration and regeneration
Case IV	34	6/28 Insufficiency and stenosis of mitral valve, stenosis of aortic valves, enlarged liver, cyanosis, extreme dyspnea, slight jaundice, no edema 7/5 Died	Hb 100, 3% normoblasts, leukocytes 7,000	No autopsy
Case V	71	3/4 Decompensated hypertension, cardiac asthma, extreme dyspnea and cyanosis, slight jaundice, hydrothorax, ascites, gangrene and edema of legs 3/10 Regression of decompensation 8/30 Many attacks of dyspnea, general condition poor 11/4 Died	3/4 1% normoblasts, leukocytes 1,300 3/10 no normoblasts	Autopsy Hypertrophy of heart, emboli of lungs, chronic nephritis

TABLE 2c—*Continued*

Patient	Age	Clinical data	Hematologic data	Remarks
Case VI	48	11/9 Insufficiency and stenosis of mitral valve, dyspnea and cyanosis, gangrene of tip of nose and both legs below knee, obturated left femoral artery and right popliteal artery, infarction in lung 11/16 Died	11/9 Hb 59, 1% normoblasts, leukocytes 22,000	Autopsy Chronic endocarditis, stenosis of mitral valve, thrombus in left heart-ear, embolic obturation of left iliac artery, infarction in lung, congestion of liver
Case VII	45	11/19 Insufficiency and stenosis of mitral valve, auricular fibrillation, decompensation of heart, infarction in lung, gangrene of tip of nose and of right foot, extreme dyspnea and extreme cyanosis bundle branch block 11/20 Died	11/19 Hb 78, 4% normoblasts, leukocytes 2,800	Autopsy Chronic endocarditis, stenosis of mitral valve, thrombus in right auricle of heart, cardiac cirrhosis of liver with regeneration, infarctions in lungs
Case VIII	40	4/14 Insufficiency and stenosis of mitral valve, auricular fibrillation, cirrhosis of liver, dyspnea, cyanosis, slight jaundice 10/14 Died	10/1 Hb 78, 1% normoblasts, leukocytes 10,600	Autopsy Chronic endocarditis of mitral, aortic and tricuspid valves, thrombus in right atrium, cirrhosis of liver
Case IX	63	11/13 Insufficiency and stenosis of mitral valve, auricular fibrillation, decompensation of heart, slight dyspnea, extreme cyanosis, infarction of lungs 12/12 General condition poor 2/5 of next year Died	11/15 Hb 88, 2% normoblasts, leukocytes 5,600	Autopsy Chronic endocarditis of mitral valve, infarctions in lungs

of decompensated valvular heart disease as well as cases of heart failure in hypertension or in coronary thrombosis. In Cabot's cases it might be argued that the normoblastosis was a result of the anemia rather than of heart disease, but in all other cases anemia played no role. The predominant factor in these nonanemic cases seemed to be a marked diminution of the oxygenation of the blood in the lungs, as evidenced during life by severe cyanosis. After death this was explained

in many cases by the finding of a thrombus in one of the auricles or ventricles of the heart and/or infarcts of the lungs. Three of Frank and Hartmann's cases had myocardial infarction with secondary formation of a mural thrombus in the left ventricle. One patient had a thrombosis of the right pulmonary artery. Two patients had severe decompensation as a result of mitral stenosis. In all our cases, thrombosis or embolism inside the heart or pulmonary arteries was found, eight times this was verified at autopsy. Apart from thrombi and emboli in the heart and lungs, the postmortem examinations showed no changes which could have caused the normoblastosis. Fatty degeneration of the liver was regularly present, cirrhosis and regeneration of bile ducts were sometimes prominent features. No extramedullary blood formation was found.

Taking into consideration Cabot's anemic cases, the conclusion is justified that peripheral normoblastosis occurs especially in those patients with heart failure who

TABLE 3

No	Age	Diagnosis	Normoblasts	Duration of life after first detection of normoblasts	Cyanosis	Dyspnea	Thrombi in heart	Infarcts in lungs
			<i>per cent</i>					
1	34	Mitral disease	5	13 days	+	++	+	+
2	56	Mitral disease	10	12 days	+	+	+	+
3	20	Mitral disease	7	1 day	—	+	+	—
4	34	Mitral disease, aortic stenosis	3	7 days	+	++	?	—
5	71	Decompensated hypertension	1	10 months	++	++	—	+
6	48	Mitral disease	1	7 days	+	+	+	+
7	45	Mitral disease	4	1 day	++	++	+	+
8	40	Mitral disease, tricuspid stenosis	1	13 days	+	+	+	—
9	63	Mitral disease	2	3 months	++	++	—	+

are either markedly cyanotic or strongly anemic, so that it appears as if anoxia is the most important cause of the normoblastosis. We are inclined, therefore, to regard the occurrence of normoblasts in the peripheral blood as an indication of an attempt on the part of the body to increase the number of circulating red cells as a result of the stimulus which anoxia exerts on the blood-forming apparatus. Apparently, normoblasts appear in the peripheral blood only when anoxia is extreme, in a degree that occurs only in the very severe forms of heart failure.

Decompensation alone does not seem to produce the phenomenon, it requires the presence of thrombi and/or infarcts in the lesser circulation. This is probably the reason why Walter, Blumgart and Volk<sup>3</sup> did not find normoblasts in the blood in their cases of congestive heart failure, as they excluded all cases with complications from their study. They noted, however, that there was an increase in reticulocytes in the peripheral blood in heart failure, which disappeared when the condition improved.

The presence of normoblasts in the peripheral blood in heart failure thus seems to indicate that the condition is complicated by mural thrombosis in the heart or

pulmonary artery, or by pulmonary emboli, or a combination of these conditions. Hence, it is easily understood why peripheral normoblastosis is a sign of such poor prognosis.

In some, but not in all cases, the normoblastosis was accompanied by a leukocytosis and (or) the presence of young precursors of the myeloid group. Some cases showed not only a peripheral normoblastosis but a distinct leuko-erythroblastic blood picture. This was most pronounced in one of Frank and Hartmann's patients who had a total white count of 21,000 and not less than 70 per cent nucleated red cells.

### SUMMARY

The authors recommend the search for normoblasts in the blood of patients with severe heart failure. When normoblasts are found, a marked interference with the oxygenation of the blood, either by pulmonary infarcts or thrombi inside the heart, is most likely to be present. It seems justifiable to consider the prognosis as very grave in these cases. This rule proved to hold even in those cases where, concomitant with an improvement in the heart failure, the normoblasts disappeared temporarily from the peripheral blood.

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# THIAMIN DEFICIENCY IN THE RHESUS MONKEY

## CLINICAL, METABOLIC AND HEMATOLOGIC OBSERVATIONS

Bj J F RINEHART, M D , L D GREENBERG, PH D , AND L L GINZTON, M D

AN IMPORTANT advance in nutritional research was the development of an essentially synthetic diet adequate for study of single deficiencies in the monkey.<sup>1</sup> It seemed most timely to restudy the vitamin deficiencies in a primate whose metabolic processes might be expected to approximate those of man most closely. This report is concerned with the study of thiamin deficiency. Seven rhesus monkeys (*Macaca mulatta*) were subjected to one or more episodes of thiamin deficiency. Observations were made on food consumption, weight, clinical behavior, thiamin metabolism and the blood picture. Finally the animals were sacrificed and detailed pathologic examinations were made. In this report the experimental method is reported together with the clinical metabolic and hematologic observations enumerated above. The pathologic findings resulting from recurrent thiamin depletion with particular reference to characteristic degenerative changes occurring in the heart muscle and severe retrogressive changes in the nuclear structures of the central nervous system have been reported<sup>2, 3</sup> and will be detailed elsewhere.

### EXPERIMENTAL METHOD

The diet used in these experiments was a modification of the M-3 diet of Waisman et al<sup>4</sup> and consisted of powdered sucrose 73, vitamin test casein (General Biochemicals) 18, Hawk and Oser salt mixture 4, and corn oil 2. Sulfited liver extract equivalent to 100 grams of Wilson Laboratories L fraction prepared according to the method of Kline and co-workers<sup>5</sup> was added to each 4 kilograms of diet. The diet was dried, granulated and following the addition of 1 per cent calcium stearate compressed into tablets weighing approximately 2 grams.\* The basal diet was fed ad libitum. The diet in pellet form had the advantage of curtailing waste and facilitating the estimation of the daily food consumption. A vitamin tablet containing daily dosages similar to those of Waisman et al was fed each day. Each vitamin tablet contained the following: nicotinic acid, 5 mg, riboflavin, 1 mg, pyridoxine hydrochloride, 1 mg, calcium pantothenate, 3 mg, choline dihydrogen citrate, 100 mg, paraaminobenzoic acid, 100 mg, inositol, 100 mg, and ascorbic acid, 25 mg, plus sufficient powdered sugar to make a tablet weighing 1.5 to 2 grams. The monkeys accepted these vitamin tablets willingly and consumed them eagerly. Control monkeys were also given 1 or 0.5 mg thiamin chloride daily. In addition the monkeys received by mouth 5 drops of vitamin A and D concentrate twice weekly and additional sulfited liver extract (equivalent to 2.5 Gm daily) twice or thrice weekly as a source of biotin and folic acid. Some animals also received 5 drops of mixed tocopherols (Napco) once a week.

During the course of these studies the weights and daily food consumption were followed carefully. Blood was taken by venipuncture at approximately weekly intervals for the determination of thiamin.

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levels and for hematological studies. At autopsy portions of several tissues were removed from each monkey and prepared for analysis of thiamin and riboflavin content. The methods used for the analysis of thiamin of blood and tissues have previously been described.<sup>9</sup> The method for the estimation of riboflavin will be reported in another article. The animals were tuberculin tested by injection with old tuberculin in the eyelids. Positive reactors were rejected. The monkeys were then placed on the purified diet with complete supplementation for one to two weeks, and control tests were carried out so that each monkey could serve as his own control. The occasional animal who failed to adapt itself to the diet or failed to gain in weight during the control period was rejected. With the exception of one monkey all the animals used weighed between 1800 and 3600 grams. The one exception was an older animal weighing 7000 grams which had previously been used in some other studies. In all, 7 monkeys were employed in this study and were subjected to one or more periods of depletion.

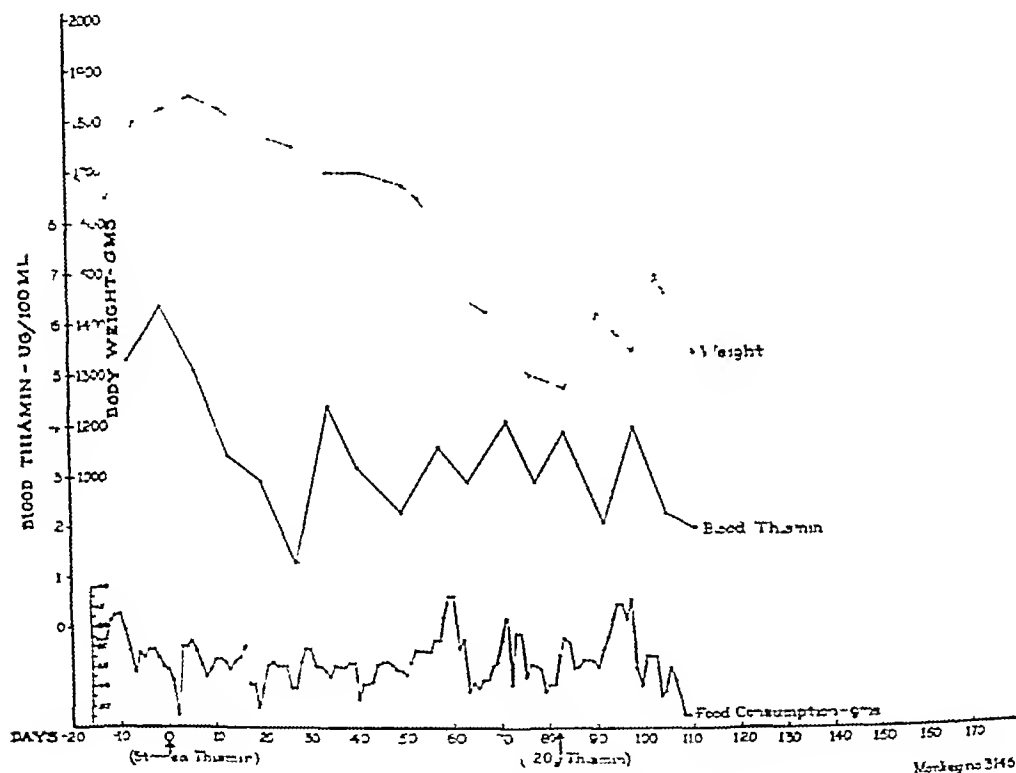


FIG. 1. THIAMIN DEFICIENCY IN MONKEY NO. 3146.  
Graph of food consumption, blood thiamin, and weight.

#### CLINICAL OBSERVATIONS

The clinical behavior of the animals was in most respects similar to that reported by Waisman and McCall.<sup>10</sup> In general the monkeys ceased gaining after two weeks on the thiamin deficient diet. This was either followed by a plateau of the weight curve for several days or by loss of weight. The weight loss was usually associated with a decreased food consumption and marked lowering of the blood thiamin as is shown in figure 1 and 2, which are representative of the changes observed in monkeys on the thiamin deficient diet. As the deficiency progressed the animals continued to lose weight, became apathetic and inactive, and weakness was evident. Finally, if the depletion period were prolonged, the animals became ataxic. Some developed prosis and tremors. Retching was observed on several

occasions The monkey would make every attempt to prevent the escape of vomitus from his mouth by trapping it in his buccal pouches and would ultimately reswallow it Convulsive movements have been observed in one or more of the animals If thiamin were administered at this stage a dramatic response was observed in twenty-four to forty-eight hours The improvement in locomotion, alertness and appetite was striking On the other hand, if the period of thiamin deprivation were not interrupted at this stage it was but a matter of a few days until the monkey was unable to sit on its perch or even stand upon the floor of the cage without difficulty

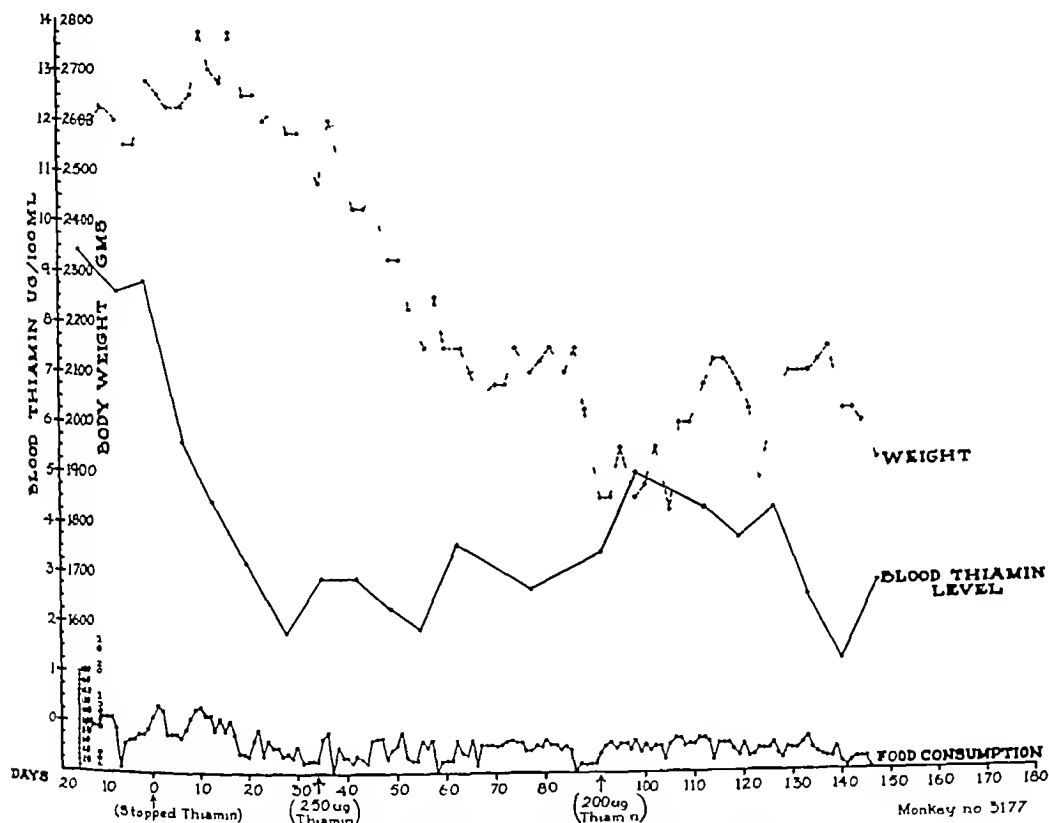


FIG 2 THIAMIN DEFICIENCY IN MONKEY NO 3177  
Graph of food consumption, blood thiamin, and weight

At times there appeared to be a paralysis of the hind legs The animal could climb or move in the cage only by the use of its forelegs Occasionally the onset of acute thiamin deficiency was so sudden that no manifestations were evident, aside from weight loss, mild anorexia and decreased activity, until the animal became ataxic, followed by a state of collapse Edema was observed in only one animal during the period of depletion However, following administration of thiamin to acutely deficient animals we have in several instances observed the appearance of edema The control animals continued to gain weight (although not as rapidly as animals we have had on our stock diet), and to remain strong and healthy during the course of the experiment We have maintained control animals on the complete diet

for a year or longer without any obvious alterations in their strength or vigor. One control animal was sacrificed and autopsied after having been on the diet for a period of six months and no pathologic changes were in evidence during either gross or microscopic examination of the tissues.

### THIAMIN METABOLISM

*Blood thiamin.* During the period while the monkeys were on the complete diet including the thiamin supplement, the blood thiamin levels ranged from 5.5 to 10 micrograms per 100 ml of whole blood. This range of values is similar to that observed by us in healthy human beings. Following withdrawal of the thiamin, and simultaneously with the first fall in weight and food consumption, the blood thiamin usually dropped to values of 4 micrograms or less. Except for some minor fluctuations these values remained low. When sufficient thiamin was administered, this was reflected in the blood level by a significant rise. The alterations of the

TABLE 1—*Thiamin Requirement of Monkeys*

Monkey no	Wt	Dose of thiamin administered	Elapsed time	Minimum requirement micrograms/kg/day
	kg	μg		
245	5	4000	36	22
69	2.75	1000	50	7.5
3192				
1st-2nd depletion	1.9	200	11	9.6
2nd-3rd depletion	1.7	500	12	24.6
3rd-4th depletion	1.7	250	8	18.3
73	2.57	2500	91	27.5
Average				15.5

blood thiamin levels are charted in figures 1 and 2. The blood thiamin levels of control animals remained well above 5 mg per 100 ml during the course of the experiment.

*Minimal thiamin requirement.* A rough estimate of the minimum thiamin requirement of the monkey can be obtained by observing the time required to redeplete an acutely deficient animal following the administration of a small dose of the vitamin. Although there is a possibility that a portion of the vitamin may pass through the gastrointestinal tract unabsorbed (if administered by mouth), or that a portion may be eliminated in the urine if the dose is too large, for this calculation the assumption is made that the full dose is retained. If the total dose is divided by the product of the elapsed time in days and the weight in kilograms, one obtains a value for the minimum daily requirement per kilogram of body weight. Calculations of this type have been carried out on 4 individual monkeys and are summarized in table 1. In the case of Monkey No. 3192 which was carried through four depletion periods, three different observations on this same animal are recorded. The values recorded show considerable variation. However, the average

value obtained is 15.5 micrograms per kilograms per day and is in close agreement with the value reported by Waisman and McCall.<sup>4</sup> It should be pointed out that this does not represent an intake adequate for normal metabolism.

**Tissue thiamin** Certain tissues were removed at the time of autopsy and subjected to thiamin and riboflavin analysis. In nearly all cases (with the exception of one) the deficient animals were sacrificed in the terminal stage of deficiency by the administration of chloroform. For purpose of comparison a control animal was sacrificed at the termination of the study. The latter was in excellent health after having been maintained on this purified diet for a period of six months. In table 2 we have summarized the results of our analyses. These data show that there is a marked lowering of the thiamin content of every tissue examined in the deficient animals.

In the case of riboflavin the heart, kidney and liver concentrations of the deficient animals were found to be slightly higher than those observed in the control

TABLE 2

Mon key no	Control (C) or defic (D)	Skeletal Muscle		Brain (cortex)		Heart (ventr)		Kidney		Liver	
		thiamin	ribo flavin	Thiamin	Ribo flavin	Thiamin	Ribo- flavin	Thiamin	Ribo- flavin	Thiamin	Ribo flavin
		$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$	$\gamma/\text{gm}$
3163	C	0.9	3.1	1.6	2.5	3.5	6.8	3.5	19.6	2.1	18.9
3175	D	0.4	—	0.3	—	0.3	6.6	0.5	17.8	0.7	24.1
3192	D	0.2	1.8	0.4	2.8	0.3	8.7	0.6	26.3	0.5	21.4
3177	D	0.3	1.7	0.5	2.5	0.3	10.0	0.9	23.1	0.7	26.8
3146	D	0.5	1.8	—	—	0.4	10.2	0.6	22.0	0.6	25.4
69	D	0.2	1.5	—	—	0.9	9.6	1.0	24.8	0.8	21.4
73	D	0.2	1.2	—	—	0.3	7.3	1.3	21.3	0.9	18.5
245	D	0.3	1.2	—	—	0.3	8.2	0.8	25.2	0.9	18.4

animal. However, this is offset by the greater concentration of this vitamin in the skeletal muscle of the control animal.

Although it is difficult to compare tissue vitamin levels of one species of mammal with those of another species since the intake is known definitely to influence the concentration of the vitamins, we can point out that the control levels found in the monkey are similar in some respects to those found in the rat and in man. Figures<sup>6</sup> obtained in our laboratory on the rat on a 20% per day level are of the same order for heart and muscle, but higher values for kidney and lower values for liver are found in the monkey. In general, rats with acute thiamin deficiency show lower figures for the tissues analyzed than those found in the monkey during the acute stage of deficiency. The control thiamin values for monkey's skeletal muscle and heart are not significantly different from those of humans who have died of accidental death. Liver and kidney of human origin have shown lower values than those found in monkeys. The tissue thiamin values recorded in the control monkey probably represent saturation values in as much as the animal had received daily thiamin in excess of its metabolic requirement.

## HEMATOLOGIC OBSERVATIONS

It is generally considered that thiamin deficiency per se has little if any influence in hematopoiesis.<sup>7 8</sup> Our data indicate a significant influence on erythropoiesis as is illustrated in figure 3. It will be seen that with depletion for thirty days there was a slight but definite reduction in the red blood cell count and hemoglobin. At forty days this was obscured presumably by dehydration. However, at this time the reticulocyte count had fallen to zero. On administration of small subcurative doses of thiamin there were definite reticulocyte responses. Interestingly this was accompanied by a fall in the red blood cell count and hemoglobin which was probably brought out by correction of dehydration. A second

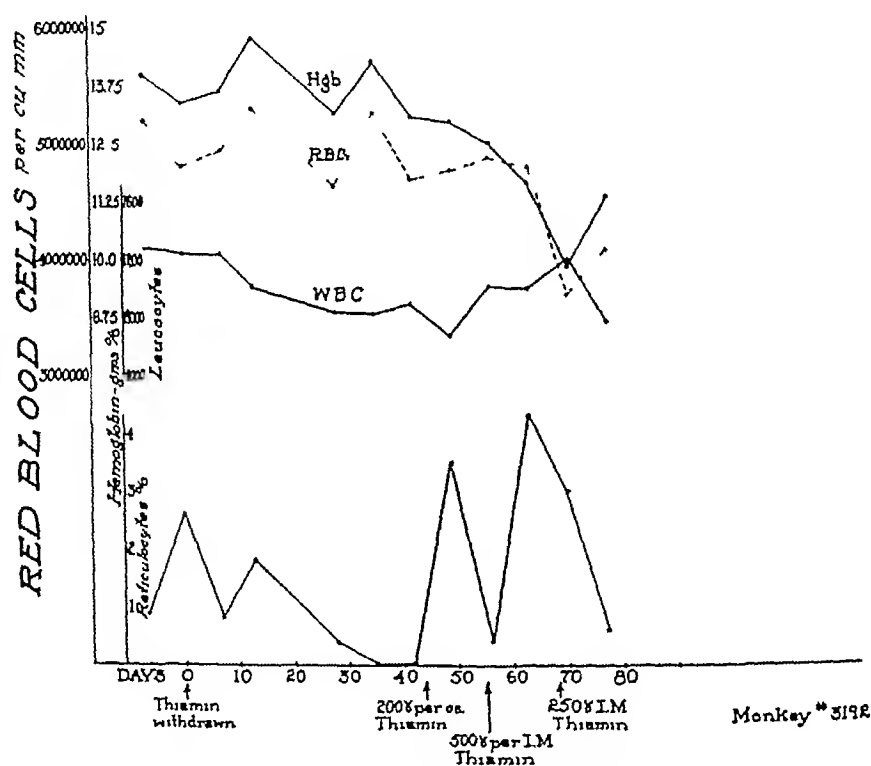


FIG 3 BLOOD IN THIAMIN DEFICIENCY IN MONKEY NO 3192

animal showed a progressive fall of the red blood cell count and hemoglobin beginning at thirty days and continuing to the termination of the experiment at fifty days. In this animal the red blood cell count fell from 4.7 million to 3.6 million and the hemoglobin fell from 13.4 grams to 8.75 grams. The reticulocyte count during the early phase of the experiment approximated 1 per cent falling to zero between thirty two and fifty days. The complete suppression of circulating reticulocytes is of particular interest and is unique in our experience, not occurring in other deficiencies of the vitamin B factors studied. The small bleedings for metabolic studies probably contributed very little to the anemia. Such anemias did not develop in control animals. The cases cited are typical of the 4 animals in which hematologic examinations were made. The conclusion seems justified that thiamin

deficiency in the rhesus monkey will cause anemia and that the mechanism is evidently due to suppression of reticulocyte formation \*

### SUMMARY

Seven rhesus monkeys were subjected to one or more episodes of acute thiamin depletion. It is clear that significant metabolic inadequacies preceded demonstrable structural changes. Diminished food consumption and weight loss were manifest about two weeks after thiamin was removed from the diet. When the deficiency was prolonged the animals became apathetic, inactive and progressively weaker. This was followed by ataxia and at times ptosis and tremors. Even in such advanced states of depletion, administration of thiamin produced dramatic improvement in locomotion, appetite and reactivity. The blood thiamin content of normal monkeys ranged from 5.5 to 10.7 per 100 ml of whole blood, values which are comparable to those of healthy human beings. Following withdrawal of thiamin the blood concentration fell to values of 4.7 or less. The tissue content of thiamin was correspondingly reduced in depleted animals. The minimum daily requirement for thiamin calculated on the basis of the time required to redeplete a deficient monkey following a small dose of thiamin was approximately 15.7 per kilogram body weight. Characteristic degenerative changes in the heart muscle and severe retrogressive changes in the nuclear structures of the central nervous system previously reported were noted. Based on careful hematologic studies in 4 animals it is concluded that thiamin is essential for normal erythropoiesis. Acute or chronic depletion results in anemia due to suppression of red blood cell formation as indicated by severe depression or absence of reticulocytes in the blood.

### ACKNOWLEDGMENT

We are indebted to Miss Mariette Quigley for the blood counts, and to Miss Ruth Johnson for assistance in thiamin assays.

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\* Since this article was written we have found a report by Fornaroli (*Arch. fisiol.* 41: 276-285, 1941) in which he records "a large reduction in reticulocytes in vitamin B<sub>1</sub> deficient rats."

# STUDIES ON HYPOPROTEINEMIA I HYPOPROTEINEMIA IN PATIENTS WITH GASTRIC CANCER, ITS PERSISTENCE AFTER OPERATION IN THE PRESENCE OF BODY TISSUE REPLETION

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*With the technical assistance of IRIS FORBES, A B*

## INTRODUCTION

**H**YPOPROTEINEMIA occurs with greater frequency in patients with gastric cancer than in patients with other neoplasms or with benign lesions of the gastro-intestinal tract. This was noted earlier by various authors,<sup>1-6</sup> and has been studied in this laboratory. Hypoproteinemia becomes more pronounced and resistant to therapy following surgical treatment for cancer of the stomach than following similar operations for benign lesions of the stomach or for cancers of other organs.

In previous studies from this laboratory, it was shown<sup>7</sup> that the preoperative administration of large amounts of protein to patients with gastric cancer renders the postoperative hypoproteinemia less pronounced. The present study revealed that hypoproteinemia, once it is established in patients with gastric cancer following operation, is persistent in spite of the administration of large amounts of protein adequate to result in significant increase of body tissue protein.

## CASE MATERIAL AND METHODS

### GENERAL PLAN OF STUDY

Patients who underwent partial gastrectomies for benign gastric ulcers and patients undergoing similar operations or exploratory laparotomies for cancer of the stomach were given high protein, high caloric feedings as soon as possible following surgery. Nitrogen balances were recorded for from 7 to 108 days and potassium, calcium, sodium and phosphorus balances were studied as well. At regular intervals, plasma volumes, plasma protein concentrations and electrophoretic plasma protein fractions were determined. It was thus possible to evaluate nitrogen retention, body tissue repletion and plasma protein regeneration, to compare the changes occurring in patients with benign gastric lesions to those observed in patients with gastric cancer and to evaluate the utilization and distribution of nitrogen in both groups.

### CASE MATERIAL

(Detailed case histories are given in the Appendix.) The initial plasma protein values of all patients are recorded in table 1.

#### *Patients with Gastric Cancer*

Three patients had cancer of the stomach which was operable, and underwent partial gastrectomy (cases 4, 5, and 6). These were 1 female and 2 males, aged 60, 46, and 50 respectively.

Seven patients had inoperable cancer of the stomach. Six of these underwent exploratory laparotomy.

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TABLE 1

Case no.	Days	Weight, kg		Plasma protein Gm/100 ml		Plasma volume		Total circulating protein		Nitrogen intake			Nitrogen output			Nitrogen retained	
		Initial	Final	Average	Initial	Final	Initial	Final	Initial	Final	Gm/day	Gm/day/kg	Total Gm	Gm/day/kg	Total Gm	Total Gm	Intake
Gastric ulcers																	
1	11	56 1	55 3	5 52	6 56	3290	2910	182 6	191 0	1 1/4	0 26	15 3	0 28	168	—	10	—6
2	10	56 8	57 7	1 85	6 66	3300	2510	160 1	169 2	28 5	0 19	22 1	0 39	22 1	—	61	31
3	10	17 7	17 5	5 62	5 66	2010	3300	114 6	186 8	—	—	—	—	—	—	—	—
Operable cancers																	
1	11	51 1	53 0	5 76	5 25	2220	2190	127 9	115 0	2 1/9	0 17	26 8	0 51	295	—	21	—8
5	12	75 0	—	6 13	6 15	3610	3060	221 3	188 2	—	—	—	—	—	—	—	—
6	18	66 1	—	5 21	6 56	4860	3520	251 7	230 9	—	—	—	—	—	—	—	—
Inoperable cancers																	
7	11 1/2	63 2	63 1	6 13	6 81	2110	2380	119 6	162 1	35 3	0 56	15 7	0 25	157	—	196	56
8	7	17 7	19 3	6 17	5 06	2210	2300	111 9	116 1/2	40 0	0 81	22 6	0 46	158	—	122	44
9	11	19 5	—	7 20	5 18	1820	1870	131 0	102 5	10 1	0 21	8 6	0 17	121	—	21	15
10	17	67 3	61 1	6 67	6 51	3190	1008	232 8	262 1	17 2	0 27	17 2	0 27	292	—	0	0
11	12	61 1	—	6 37	5 80	3450	3450	219 8	200 1	17 8	0 28	11 9	0 19	113	—	71	33
Inoperable cancers—long term studies																	
12	108	53 6	57 3	6 06	5 11	3100	3750	187 9	191 6	27 9	0 19	21 2	0 42	2615	—	402	13
13	26	69 5	69 5	5 32	6 36	5160	1220	271 5	268 1	29 6	0 13	19 5	0 28	506	—	26 1/2	34

\* 1 month of study, days

† Beginning of study, days after operation

‡ Nitrogen balance for 10 days only, plasma protein change in 14 days



(cases 7, 8, 9, 10, 11 and 13) These were two females and four males, aged 69, 47, 52, 49, 70, and 64, respectively

One patient (case 12) was found to have a resectable cancer of the stomach which was removed, but firm, large lymph nodes were felt in the portal region and in the omentum and were not removed. This man was 65 years old.

### Control Subjects

Three patients with benign gastric ulcers were studied (cases 1, 2, and 3). These were three males, aged 51, 45, and 53. This control group was kept small because previous work by Co Tui et al.<sup>8</sup> had clearly shown that patients with gastrectomy for ulcers on a high nitrogen intake regenerate plasma protein within two weeks following operation and our findings confirmed this observation.

TABLE 2—*Routine for Administration of Protein Hydrolysate in Patients Following Gastrectomies as it Was Used at the Memorial Hospital at the Time of this Study*

Time	Hydrolysate per feeding	Frequency of feeding	Water to be added	Remarks	Parenteral 5% dextrose
	cc	hr			cc
Day of op					
6 hrs p o	—	—	30 cc q 1 hr	Alternate as required	
11 hrs p o	30	q 2	30 cc q 1 hr	Same	
First day	Same	Same	Same		2500
Second day	Same	Same	Same		2500
Third day	30	q 1	30 cc q 1 hr	To rinse tube	1500
Fourth day	60	q 2	75 cc q 2 hrs	To rinse tube	1500
Fifth day	60	q 2	45 cc q 2 hrs, 50 cc boiled milk added	After hy- drolysate	1000

Sixth and seventh day as above. Reduce parenteral fluid according to patient's ability to swallow small amounts of water by mouth. Remove stomach tube on eighth day and start patient on routine seventh day gastrectomy diet.

### Methods

1. *Alimentation* Some of the patients had orojejunal feeding tubes inserted at operation and left in situ for as long as seven days postoperatively. Some had external jejunostomy tubes for feeding purposes. Unless otherwise specified, casein hydrolysate Squibb\* was used as a source of nitrogen in all cases. In general, the plan included the administration of 0.6 Gm of nitrogen per day per Kg of body weight in a mixture of the hydrolysate in a 10 per cent dextrose solution with 60 Gm of Amphojel. This feeding mixture was administered by the jejunal tube and/or by mouth and the total daily volume was adjusted to 720 ml. A feeding schedule given in table 2 was followed as closely as possible. After the first post-operative period this regimen was supplemented by the usual gastrectomy hospital diet in amounts measured in the metabolic kitchen and analyzed in the laboratory for nitrogen content. The caloric content of the total diet, including parenteral alimentation, was maintained at a level of approximately 1800 calories per day (except on the first, and sometimes, the second postoperative day when it was lower).

\* The hydrolysate was provided by the courtesy of the Squibb Co.

Plasma and blood transfusions were given in all cases on the day of operation and thereafter as sparingly as possible to avoid interference with the observations on protein regeneration. Parenteral fluid intake was standardized as closely as possible, 1000 ml of physiologic saline solution and 300 ml of dextrose in water on the second and only rarely on the third postoperative day. All parenteral intake, as well as other alimentation, was carefully measured and recorded. The patients received a daily intramuscular injection of a vitamin B complex preparation\* throughout the duration of the metabolic study. Other medication was given as indicated. The patients usually were allowed up on the second or third postoperative day, but this was not standardized. The 2 patients on whom long-term metabolic studies were carried out for 26 and 108 days were placed on a constant basic diet which was the same every day.

In addition, to maintain the desired high nitrogen content, these patients received part of their nitrogen intake in the form of protein hydrolysate (Casein Hydrolysate, Squibb) and in the form of native protein (milk protein as Delcos, Sharp and Dohme, and Lactalbumin Squibb in unhydrolyzed form).

2. *Collection of specimens* Urines were collected in 24-hour specimens, preserved at pH 3 with acetic acid, and kept in the refrigerator without preservative. Vomitus and gastric aspiration fluid were collected in 24-hour samples and kept in the refrigerator without preservative.

### 3. *Methods of Analysis*

*Urine* Urinary nitrogen was measured by a micro-Kjeldahl method. Potassium and sodium were measured by flame photometry<sup>9</sup>, phosphorus was determined by Fiske and Subbarow's method,<sup>10</sup> and calcium by the method of Schohl and Pedley.<sup>11</sup> Creatinine determinations<sup>12</sup> were used to evaluate the accuracy of the collections.

*Stools* A commercial homogenizer was used for the thorough mixing of stools which were then made up to a standard volume by the addition of distilled water. Nitrogen was measured by a micro-Kjeldahl method, and minerals were determined in dry ashed aliquots by the same methods used in the urine.

*Blood* Plasma protein was measured by a micro-Kjeldahl method and corrected for nonprotein nitrogen. The plasma protein components were estimated by electrophoresis by Dr. M. L. Petermann.<sup>13</sup> Plasma volumes were measured by the use of Evans blue.<sup>14</sup>

## RESULTS

### *Body Tissue Repletion Table 1*

*Repletion within one month after operation* In only 2 of the 3 ulcer cases studied were nitrogen balance studies done. The third case is included because, while nitrogen output was not measured, the nitrogen intake was known and was at least as high as in the two others. As previously shown by Co Tui et al.<sup>8</sup> and by Reigel,<sup>15</sup> positive nitrogen balance† was obtained in both cases, the usual postoperative nitrogen loss, therefore, was offset by adequate nitrogen utilization. This was achieved on intakes of 0.26 and 0.49 Gm of nitrogen per Kg per day, or considerably less than had been planned originally. All patients with gastric cancer were in nitrogen balance or had positive nitrogen balance 7 to 20 days following operation. There was essentially no difference between those having undergone exploratory laparotomy and the one case with gastric resection in which nitrogen balance studies had been done (case 4). The least marked nitrogen retention actually occurred in the latter case. The nitrogen intake in the cases with cancer ranged from 0.2 Gm per Kg per day to 0.81 Gm per Kg per day, with an average of 0.43 Gm per Kg

\* Beta Symplex was used (Winthrop) which has the following composition: thiamin, 10 mg; riboflavin, 5 mg; calcium pantothenate, 5 mg; niacinamide, 50 mg.

† A patient is considered to be in nitrogen balance when the loss of nitrogen exceeds the intake by less than 0.01 Gm per Kg per day.

per day. An average of 26 per cent of the ingested nitrogen was retained as compared to 12 per cent (31 and -6 per cent) in the ulcer group.

*Repletion more than one month after operation.* Studies of nitrogen and mineral balances were made in 2 cases of gastric cancer following exploratory laparotomy (case 12) and following palliative gastrectomy (case 13) during periods of 26 and 108 days and starting 12 and 11 days after operation. These showed marked nitrogen retention 402 Gm. or 0.065 Gm. per Kg. per day for 108 days, equal to 13 per cent of the intake, and, 264 Gm. or 0.15 Gm. per Kg. per day or 34 per cent of the intake.

TABLE 3—Comparison between Theoretic and Actual Nitrogen Intake and Changes of Circulating Protein

Case no	Type case	Theoretic nitrogen requirement*	Nitrogen actually received	Protein observed change	Protein, calculated change†	Difference
				gm	gm	gm
1	Ulcer	323	158	+9.3	-2.1	+11.4
2	Ulcer	395	285	+9.1	+12.7	-3.6
3	Ulcer	261	—	+71.7	—	—
4	Operable cancer	241	274	-13.0	-4.3	-8.7
5	Operable cancer	468	—	-33.0	—	—
6	Operable cancer	473	—	-23.7	—	—
7	Inop. cancer	258	353	+13.6	+41.0	-27.4
8	Inop. cancer	163	280	-28.5	+25.4	-53.9
9	Inop. cancer	246	142	-27.6	+4.4	-32.0
10	Inop. cancer	205	292	+29.3	0	+29.3
11	Inop. cancer	262	214	-19.9	+14.8	-44.7
12	Inop. cancer, long-term study	654	3017	-1.0	+83.0	-84.0
13	Inop. cancer, long-term study	564	770	-7.1	+55.0	-62.1

\* Theoretic Nitrogen Intake means the amounts of nitrogen required for correction of the plasma albumin deficit (Assumed normal albumin 4.6 Gm./100 ml. All calculations are based on values obtained by the Howe method because this method was the one used in evaluating the proportion of albumin to the remaining body protein, ref. Elman, R. Protein deficiency in surgical patients and its correction. *J. Am. Diet. A.* 18: 141-144, 1942. The total calculated nitrogen requirement includes 4 Gm. for daily maintenance during the period of study, the tissue protein loss calculated from the albumin lost and assumes a loss of 50 per cent of the ingested nitrogen (above the maintenance requirement of 4 Gm. per day).)

† The calculated change of plasma protein is based on the amounts of nitrogen retained or lost, assuming that plasma protein represents one thirtieth of the total body protein.

in 26 days. There was concomitant retention of phosphorus and potassium in the proportions in which these minerals are known to exist in protoplasm. In these two instances, the ability of these patients to build body tissue is thus well demonstrated.

#### Plasma Protein Regeneration Table 3

*Regeneration within one month after operation.* In all patients with gastrectomy for benign ulcers, an increase in circulating plasma protein occurred promptly. The extent of this increase varied widely in these 3 patients and was predominantly in

the globulin fraction in one. The increase of the circulating plasma protein was of the same order of magnitude as that observed by Co Tui under similar conditions and not to be ascribed to changes of plasma volume alone (table 1). In all patients with gastric cancer except two (case 10 and case 7), there was a decrease of circulating plasma protein during the period of study. In case 10, the increase observed follows a rise of the plasma volume more marked than in most of the other cases and thus may be only illusory. In case 7, there was an actual increase, entirely caused by a rise of plasma globulin.

TABLE 4—*Changes in Globulin and Albumin as Determined by Electrophoresis and Plasma Volume Measurement (Initial Values at Beginning of Study, Final Value at End of Period—Stated in Last Column)*

Case no	Type case	Change of plasma volume	Albumin†			Globulin†			Days
			Initial	Final	Change	Initial	Final	Change	
		%			%				
1	Benign ulcer	-11.5	80.0	81.5	+2.0	101.5	100.4	+7.9	11
2	Benign ulcer	-23.0	69.5	78.9	+13.5	90.6	90.3	-0.3	10
3	Benign ulcer	+6.2	46.4	60.0	+29.3	68.0	126.5	+86.0	10
4	Operable cancer	-1.4	57.2	43.6	-23.8	70.6	71.3	+0.4	11
5	Operable cancer	-15.5	74.7	57.5	-23.0	146.6	130.6	-10.9	12
6	Operable cancer	-27.6	106.0	91.9	-13.3	149.0	139.0	-6.7	18
7	Inoperable cancer	-2.7	70.2	64.5	-8.1	79.3	97.5	+23.3	10
8	Inoperable cancer	+2.7	*	*	*	*	*	*	7
9	Inoperable cancer	+2.7	53.3	37.4	-28.1	74.0	62.3	-15.8	14
10	Inoperable cancer	+14.6	86.5	101.8	+17.7	146.2	160.3	+10.0	7
11	Inoperable cancer	0	83.1	65.2	-21.7	136.7	134.9	-1.3	12
12	Inoperable cancer— long-term study	+21.0	81.9	90.8	+10.9	106.0	101.0	-4.7	108
13	Inoperable cancer— long-term study	-18.4	121.5	119.0	-2.85	155.0	149.5	-3.6	26

\* Only total protein was determined

† Refers to total circulating fractions

*Regeneration more than one month after operation.* The changes of circulating plasma protein in these two cases following 26 and 108 days of postoperative high protein feeding were insignificant and probably within the range of technical errors.

#### *Comparison of Theoretic and Actual Plasma Protein Increases Table 4*

Studies by Weech<sup>16</sup> in dogs showed that the plasma albumin represents  $\frac{1}{10}$  of the body protein and by transferring this assumption to man, one can estimate the amounts of circulating albumin which theoretically should be formed from a given amount of retained food protein. Applying these relationships, Elman<sup>17</sup> has devised a calculation to evaluate the nitrogen need of a depleted individual from his plasma albumin concentration. The further assumption may be made that albumin represents most of the plasma proteins lost in protein depletion and that thus, the total protein may be substituted for albumin in such cases. These rough theoretic figures demonstrate that on the whole the discrepancy between the actual and the

calculated figures is much wider in the group of cancer patients than in the two ulcer cases and that it becomes extreme in the long-term studies

### DISCUSSION

Hypoproteinemia continues in gastric cancer patients after operation in spite of adequate body tissue repletion

Causes of hypoproteinemia are (1) inadequate formation, (2) increased utilization or destruction, or (3) abnormal distribution of circulating proteins. Inadequate formation may be due to (a) insufficient protein intake, (b) excessive nitrogen loss, or (c) specific defect in serum protein synthesis. In patients with gastric cancer who show hypoproteinemia before operation, insufficient intake and excessive loss of nitrogen have been eliminated by earlier studies.<sup>4-5</sup> The present investigation supplies evidence that inadequate intake and excessive loss of protein are not responsible for the persistence of the hypoproteinemia in gastric cancer patients after operation.

One concludes that either inadequate fabrication or distribution is responsible for hypoproteinemia in gastric cancer. The defect is probably nonspecific, as similar observations have been made in patients with tuberculosis.<sup>18</sup> It may be related to the gastro-intestinal tract, to the liver, or to the adrenal cortex.

Further studies are in progress to determine if the hypoproteinemia is due to decreased synthesis, to increased utilization or destruction, or to abnormal distribution of proteins.

### APPENDIX

#### *Summaries of Clinical Histories of the Patients Studied*

##### CASE 1

(B J 81337-11) colored male of 39 years. Admitted 6-25-46, discharged 7-9-46

*History* Anorexia for 2 years. Pain in midepigastrium for 2 months before admission. 13 lbs weight loss in 2 years. No vomiting, no hard stools.

*Physical examination* Tenderness in epigastrium on deep pressure, no other findings.

*X ray examination* Gastric ulcer, lesser curvature.

*Laboratory data*\* Hemoglobin 82%, RBC 4,800,000 WBC 6,100, normal differential count. Urine negative. Serum chlorides 95-105 meq/l. Serum bilirubin 1.0-1.4 mg/100 ml. Blood urea nitrogen 12-20 mg/100 ml.

Gastric analysis† Free HCl 0, 0, 22, 24, 15

Total HCl 12, 18, 35, 36, 30

*Operation* Partial gastrectomy 6-24-46

*Pathology* Chronic peptic ulcer

*Course* Uneventful postoperative recovery. Discharged 15 days postoperatively, asymptomatic since

##### CASE 2

(M P 81622-42) white male of 45 years. Admitted 5-19-46, discharged 6-5-46

*History* Preprandial pain 7 years ago, successfully treated by dietary measures. Recurrence one year ago, unrelieved by diet. In the 4 months before admission, frequent vomiting. Only 4-5 lbs weight loss

\* Data included in the paper are not repeated in the appendix

† First samples before 0.5 mg of histamine, following samples every 15 minutes thereafter. Acid in ml of N/10 NaOH

*Physical examination* Negative

*X-ray examination* Duodenal ulcer

*Laboratory data* Hemoglobin 89-103%, RBC 4,100,000-4,600,000 WBC 4,000-8,300, normal differential count Urine negative Serum chlorides 94-102 meq /l Serum bilirubin 1.1 mg /100 ml Fasting blood sugar 89 mg /100 ml Blood urea nitrogen 16.3-26.4 mg /100 ml

*Gastric analysis* Free HCl 25, 40, 60, 55, 50

Total HCl 44, 48, 64, 66, 60

*Operation* Partial gastrectomy, 5-22-46

*Pathology* Gastric ulcer, scars in duodenum, fibrous adhesions

*Course* Uneventful recovery Asymptomatic in April 1947

### CASE 3

(F H 82072-17) white male of 53 years Admitted 6-25-46, discharged 7-17-46

*History* From 18 months to 1 year before admission pain after eating Weight loss in that period 25 lbs Vomiting for about 4 weeks

*Physical examination* Negative

*X-ray examination* Crater of  $\frac{3}{4}$  inches in lesser curvature, probably benign ulcer

*Laboratory data* Hemoglobin 81%, RBC 3,700,000-4,500,000 WBC 5,000-15,000 Urine negative Serum chlorides 96-102 meq /l Serum bilirubin 0.7-1.3 mg /100 ml Fasting blood sugar 87 mg /100 ml Blood urea nitrogen 11.9-20 mg /100 ml

*Gastric analysis* Free HCl 55, 32, 28, 31, 35

Total HCl 28, 50, 65, 78, 58

*Operation* Partial gastrectomy, 7-2-46

*Pathology* Chronic peptic ulcer (gastric)

*Course* Patchy pulmonary infiltration on eighth postoperative day with moderate elevation of temperature Responded well to penicillin Asymptomatic since then

### CASE 4

(K H 82018-34) white female of 60 years Admitted 6-27-46, discharged 7-19-46

*History* Abdominal pain of 2 months duration and weight loss of 20 lbs in 4 months before admission

*Physical examination* B P 160/85, soft systolic aortic murmur Rales throughout chest, emphysematous thorax Mass and tenderness in left upper quadrant Difficult examination because of failure to relax abdominal wall

*X-ray examination* Advanced gastric carcinoma of the body of the stomach and the pyloric region

*Laboratory data* Hemoglobin 63-88%, RBC 3,800,000-4,200,000 WBC 4,400-16,800 Urine negative Serum chlorides 93-106 meq /l Serum bilirubin 0.6-1.2 mg /100 ml

*Gastric analysis* Free HCl = 0

Total HCl 5, 4, 4, 6, 6

*Operation* Subtotal gastrectomy (4/5), Hoffmeister anastomosis 7-1-46 No liver metastasis or peritoneal implants found

*Pathology* Diffuse gelatinous adenocarcinoma, extension to the fat about nodes Lymph nodes proper were clear

*Course* Uneventful recovery Patient has left town, no follow-up recorded

### CASE 5

(S S 82300-55) white male of 46 years Admitted 7-15-46, discharged 8-24-46 Readmitted for closure of colostomy 9-30-46, discharged 10-14-46

*History* 3 months persistent epigastric pain following fall from horse Lost 60 lbs in 4 months and noticed progressive weakness

*Physical examination* Essentially negative

*X-ray examination* No gastro intestinal examination

*Laboratory data* Hemoglobin 64-91% RBC normal WBC 9,000-15,000 normal differential count

Urine negative, except for occasional 1+ albumin Serum chlorides, 97-103 meq /l Serum bilirubin 0.6-4.8 mg /100 ml Serum cholesterol total 141, free 52, esters 89 mg /100 ml Fasting blood sugar 99 mg /100 ml Blood urea nitrogen 11.3-25 mg /100 ml

Gastric analysis Free HCl = 0

Total HCl 26, 10, 7, 10, 15

*Operation* No metastasis seen in liver, lymph nodes or peritoneum. A large tumor involving the greater gastric curvature and extending into the transverse colon and the omentum was found. Subtotal gastrectomy and resection of the medial portion of the transverse colon and a large portion of the omentum was performed. A Mikulicz colostomy was done (7-19-46). On 8-8-46, patient developed acute intestinal obstruction and a number of fibrous adhesions were dissected at an emergency laparotomy. The patient recovered and was discharged. On 10-4-46, colostomy was closed.

*Pathology* Adenocarcinoma, Grade III, invading entire thickness of gastric wall and extending into serosa of colon.

*Course* Patient now in good health.

#### CASE 6

(M. S. 82126-53), white male of 50 years. Admitted 6-30-46, discharged 7-22-46.

*History* Midepigastric pain for 7 months, with 11 lbs weight loss.

*Physical examination* Negative.

*X-ray examination* Carcinoma of lesser curvature, near cardia.

*Laboratory data* Hemoglobin, 48-87%, RBC 2,300,000-3,000,000 WBC 3,700-9,600, normal differential count. Urine negative. Serum chlorides 98-108 meq /l. Blood urea nitrogen 12.1-36.8 mg /100 ml.

Gastric analysis Free HCl 10, 25, 30, 55, 60

Total HCl 20, 40, 38, 75, 80

*Operation* No metastasis seen in liver, lymph nodes or peritoneum. Subtotal gastrectomy.

*Pathology* Adenocarcinoma of stomach.

*Course* Uneventful recovery.

#### CASE 7

(B. J. 80928-8), white male of 69 years. Admitted 3-21-46, discharged 4-8-46.

*History* Weight loss of 6 lbs in 3 months and loss of appetite.

*Physical examination* Negative.

*X-ray examination* Large mass in pars media of stomach.

*Laboratory data* Hemoglobin 75-85%, RBC 3,700,000-4,600,000 WBC 6,100-10,200. Normal differential count. Urine negative. Serum chlorides 100-108 meq /l. Serum bilirubin 0.7 mg /10 ml. Serum cholesterol total 197 free 61, esters 136 mg /100 ml. Fasting blood sugar 88 mg /100 ml. Blood urea nitrogen 12.4-13.2 mg /100 ml.

Gastric analysis Free HCl 0, 0, qns,\* qns, 0

Total HCl 24, 7, qns, qns, 8

\* Insufficient quantity.

*Operation* Laparotomy 3-28-46. A large mass was found involving the stomach, extending along both curvatures. Numerous metastatic nodules in liver, gall bladder, and nodes along aorta. Inoperable case.

*Pathology* Metastatic adenocarcinoma.

*Course* Complicated by hypoproteinemia (hypochloremia and edema). Patient was discharged, no follow-up notes.

#### CASE 8

(G. Y. 80364-96), white male of 47 years. Admitted 1-27-46, discharged 2-17-46.

*History* Past history of glycosuria. Negative urine at time of admission. Weight loss of 60 lbs in 18 months. Epigastric fullness and anorexia for 12 months.

*Physical examination* Evidence of weight loss, large abdominal mass

*X-ray examination* Large mass in region of cardia and larger curvature

*Laboratory data* Hemoglobin 58-95%, RBC 3,000,000-4,600,000 WBC 5,000-9,400 Normal urine  
Serum chlorides 101-105 meq /l Serum bilirubin 0.5-0.8 mg /100 ml Serum cholesterol total 121-250,  
free 42-71, esters 79-179 mg /100 ml Blood urea nitrogen 12.0-28.4 mg /100 ml

*Gastric analysis* Free HCl = 0

Total HCl 20, 10, 12, 8, 10

*Operation* Laparotomy 2-5-46 Large tumor mass, involving gastric cardia and greater portion of duodenum, as well as mesentery, spleen, pancreas, and lymph nodes Inoperable External jejunostomy

*Pathology* No biopsy material taken

*Course* Patient died 3-26-46 No necropsy

#### CASE 9

(B L 80901-6) white female of 52 years Admitted 3-19-46, discharged 4-9-46

*History* Dull abdominal pain for 4 months, small weight loss

*Physical examination* Negative

*X-ray examination* Polypoid infiltrating cancer of distal segment of stomach

*Laboratory data* Hemoglobin 65-82%, RBC 3,200,000-3,500,000 WBC 7,200-7,600 normal differential count Urine 4+ sugar, acetone 1+ (probably after glucose infusion All subsequent urines negative) Serum chlorides 91-103 meq /l Serum bilirubin 0.7 mg /100 ml Fasting blood sugar 91-100 mg /100 ml Blood urea nitrogen 8.5-20 mg /100 ml No gastric analysis reported

*Operation* Laparotomy and external jejunostomy 3-26-46 Large mass involving stomach, extension into lesser and greater omenta, multiple metastases in liver (biopsy material taken)

*Pathology* Metastatic adenocarcinoma

*Course* No follow-up notes

#### CASE 10

(S S 81726-51) white male of 49 years Admitted 5-28-46, discharged 6-26-46

*History* Weakness of 3 months duration and postprandial epigastric pain and tarry stools of 1-month duration No evidence of weight loss

*Physical examination* Abdominal mass 8 cm in diameter

*X-ray examination* Carcinoma of antrum of stomach

*Laboratory data* Hemoglobin 51-81%, RBC 2,500,000-3,800,000 WBC 5,300-14,000, normal differential count Urine negative Serum chlorides 95-104 meq /l Serum bilirubin 0.6 mg /100 ml Serum cholesterol total 141, free 52, esters 89 mg /100 ml

*Gastric analysis* Free HCl = 0

Total HCl 15, 8, 10, 12, 12

*Operation* Laparotomy and exclusion gastroenterostomy, 6-7-46 Large tumor of gastric antrum adherent to pancreas, and meso-sigmoid and colic vessels Numerous metastases in liver Firm lymph nodes

*Pathology* None reported

*Course* No follow up notes

#### CASE 11

(G G 82040-21) white female of 70 years Admitted 6-23-46, discharged 7-20-46

*History* Nocturia due to cystocele Marked weight loss (70 lbs) in 5 months before admission Anorexia and heart burn for one year prior to admission Negative gastro-intestinal x-ray examination some months before admission

*Physical examination* Mass of 10 cm diameter in mid abdomen

*X-ray examination* Gastric carcinoma

*Laboratory data* Hemoglobin 61-94% RBC 3,700,000-4,400,000 WBC 5,500-11,500 normal differential count Urine essentially negative Serum chlorides 90-102 meq /l Serum bilirubin 0.5-0.6 mg /100 ml Blood urea nitrogen 12.8-36.4 mg /100 ml

*Gastric analysis* Free HCl = 0

Total HCl 20, 10, 10, 16



*Operation* Laparotomy, 6-28-46 Extensive carcinoma with metastasis to nodes and liver

*Pathology* Adenocarcinoma, Grade III

*Course* Convalescence complicated by bronchial pneumonia

### CASE 12

(K R 83598-116) white male of 65 years Admitted 11-10-46, discharged 6-2-47

*History* Weight loss during one year preceding operation of about 30 lbs Several bouts of dark black stools and rectal bleeding

*Physical examination* Negative

*X-ray examination* Filling defect involving almost the entire pyloric portion

*Laboratory data* Hemoglobin 8.4-5.4%, RBC 1,190,000-5,000,000 WBC 4,700-8,800, normal differential count Urines negative Serum chlorides 92-104 meq/l Serum bilirubin 0.62 mg/100 ml Thymol turbidity 1.85 ml Cephalin flocculation 24 hours, negative Hippuric acid excretion 1.5 Gm Bromsulfalein retained in blood, 30 minutes, 2%, 45 minutes, 2% Serum cholesterol total 104, free 36, esters 68 mg/100 ml Blood urea nitrogen 16.3-22 mg/100 ml

*Gastric analysis* Free HCl = 0

Total HCl 20, 22, 28, 50, 34

*Operation* On 11-25-46, a large bulky lesion was found in the greater curvature of the stomach, a few nodes were palpated in the gastro-hepatic ligament The tumor was removed, and a gastro-jejunostomy was done Liver and peritoneum free of metastases

*Pathology* Gelatinous adenocarcinoma, Grade III, extensive lymphatic permeation, metastases to nodes

*Course* Essentially uneventful postoperative course This study was continued up to the 205th postoperative day <sup>19a b</sup> The patient died of carcinomatosis 66 weeks after operation Reviews of additional articles bearing on this subject, which have appeared since this paper was submitted, are noted in the references <sup>19a b</sup>

### CASE 13

(S J 109237-4) white male of 64 years Admitted 8-28-46, discharged 10-14-46

*History* Weight loss of 40 lbs in 4 months before admission Hematemesis one month before admission, epigastric fullness

*Physical examination* Mass in epigastrium

*X-ray examination* Polypoid carcinoma of stomach (fundus and body)

*Laboratory data* Hemoglobin 46-76%, RBC 2,200,000-3,400,000, Hematocrit 18-37% WBC 4,500-10,600, normal differential count Normal urine Serum chlorides 108 meq/l Serum bilirubin 1.2 mg/100 ml Fasting blood sugar 107 mg/100 ml Blood urea nitrogen 15.9 mg/100 ml

*Gastric analysis* Free HCl = 0

Total HCl qns, 14, 14, 12, 10

*Operation* 9-9-46 Large tumor from cardia through antrum along lesser curvature Liver studded with metastases Exploratory laparotomy, biopsy from liver metastases

*Pathology* Gelatinous adenocarcinoma

*Course* Died Dec 1946

### SUMMARY AND CONCLUSIONS

1 The existence of hypoproteinemia in patients with gastric cancer has once again been observed

2 The intractability of this type of hypoproteinemia in the postoperative phase to treatment with high protein diets and in the presence of positive nitrogen balance has been demonstrated

3 Long-term studies in two patients, for 28 and 108 days respectively, suggest that the persistence of hypoproteinemia in patients with gastric cancer in positive

nitrogen balance is probably not due to a marked degree of depletion of tissue protein stores alone. These patients retained more protein than would have been necessary to replenish depleted tissues.

4 It seems more likely that while such patients are capable of tissue protein synthesis, they fail to shift new protein into the blood stream.

#### ACKNOWLEDGMENT

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# STUDIES OF PHOSPHORUS METABOLISM IN MAN II A STUDY OF THE PERMEABILITY OF THE HUMAN ERYTHROCYTE TO INORGANIC PHOSPHATE IN VITRO BY THE USE OF RADIOACTIVE PHOSPHATE ( $P^{32}$ )

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*With the technical assistance of MARY KENDRICK, B S*

IT IS A distinct pleasure to dedicate the first published\* of a series of articles from this laboratory on the use of radio isotopes in human physiologic studies to Dr George R Minot. It was under his direction that our venture into this field was undertaken.

Radio phosphorus has been used in the examination of various phases of phosphorus metabolism and the results have been summarized by Hevesy<sup>1, 2</sup> and Kamen.<sup>3</sup> Most of the data presented in the literature were obtained in animals, and by use of dosages of isotopic  $P^{32}$  in excess of what may be considered tracer amounts. Human studies on the metabolism of  $P^{32}$  in tracer amounts are few in number, most of the interest of previous authors having been directed to the therapeutic uses of the isotope. Since normal metabolic processes may be deranged by larger than tracer doses of  $P^{32}$ , it seemed of value to investigate the metabolism of phosphorus in the human body using  $P^{32}$  as a tracer in doses low enough to cause no demonstrable metabolic disturbance. These studies will be presented elsewhere.<sup>4</sup> The use of  $P^{32}$  in such concentrations immediately imposes problems of revisions of methods and further considerations of accuracy of counting technics. These will be described fully in another communication.<sup>5</sup>

The first consideration in the attack on phosphorus metabolism was the resolution of the controversy of phosphorus exchange between cells and plasma of the circulating blood. The present communication is concerned with the distribution of phosphorus between red cells and plasma in in vitro studies of the phosphorus exchange between these two media. Eisenmann et al.,<sup>6</sup> have shown in an in vitro system to which inorganic phosphate together with  $P^{32}$  were added to human whole blood, that at 38 C phosphate entered the red cells freely, while at 7 C the exchange was minimal. In their experiments 5 to 101 mg phosphorus were added to 100 ml of whole blood. They also stated that, under their experimental conditions, radio phosphorus accumulated in the cells out of proportion to the amount of inorganic phosphate present. Their observations were made on whole blood and plasma and the cell concentrations of phosphorus compounds were calculated using hematocrits.

In the present communication no phosphate was added except that represented by the  $P^{32}$ , which was negligible, since the specific activity of the isotopic sodium

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\* No. 1 of this series<sup>4</sup> is in preparation.

phosphate used was very high. The *in vitro* studies presented in this paper have been extended to similar studies *in vivo* in man which will be presented elsewhere.<sup>4</sup>

## METHODS

Blood from normal human subjects was collected in bottles containing dried potassium-ammonium oxalate solution which was prepared by dissolving 3 Gm of ammonium oxalate and 2 Gm of potassium oxalate by dissolving in distilled water to a volume of 100 ml. For each ml of blood, 0.04 ml of the anticoagulant was used. Such an oxalate mixture caused no change in cell size.  $P^{32}$  in the form of sodium phosphate solution was added to the whole blood, the volumes of the radio-active phosphate solution varying from 0.58 to 2.08 ml per 100 ml of whole blood, with activities of  $P^{32}$  varying between 900 and 12,000 counts per second as registered on our counter. Samples of the radioactive phosphate solution added were utilized as standards for each experiment, and were counted at intervals throughout the experiments to correct for the decay of  $P^{32}$ . Counting was performed with a Geiger-Mueller tube with fixed geometry, and the totalizing was accomplished by the use of an autoscaler.† The background count in this laboratory averaged 0.23 counts per second. In none of these experiments was there any count lower than ten times the background. In all experiments, samples were removed for the determination of the hematocrit about five minutes after the addition of  $P^{32}$  to the blood. In this same initial sample, chemical determinations of total, total acid soluble and inorganic phosphorus were made in both plasma and red blood corpuscles. In two of the experiments, the  $P^{32}$  content of the total phosphorus of plasma and cells was also measured in the initial blood sample.

The blood was then allowed to stand in contact with  $P^{32}$ , with frequent gentle shaking, for a period of four hours. In three cases this incubation took place at a temperature of 37.5°C, and in the fourth instance the blood was kept for four hours at 7°C. At the end of four hours, the hematocrit was again determined, total, total acid soluble and inorganic phosphorus were determined chemically in plasma and red blood cells, and the  $P^{32}$  concentration was measured in all these phosphate fractions.

Blood samples were centrifuged for ten minutes at 2000 rpm to separate the plasma from the red blood corpuscles. The plasma was drawn off as completely as possible, and the blood cells were washed once with cold 0.9 per cent sodium chloride solution. The washed cells were centrifuged, and the supernatant saline drawn off and discarded. The red blood corpuscles were then frozen at -20°C in order to produce hemolysis and to prevent any hydrolysis of organic phosphorus complexes. For analysis the frozen cells were thawed and diluted with the amount of distilled water required for each determination. The exact techniques employed are described elsewhere.<sup>5</sup>

The total phosphorus of both plasma and red blood cells was determined by the colorimetric molybdate method of Fiske and Subbarow<sup>7</sup> preceded by digestion with sulfuric and nitric acids. Total acid soluble and inorganic phosphorus were determined by Fiske and Subbarow's method in trichloroacetic acid filtrates prepared from plasma and red blood corpuscles.

To determine the total amount of radioactive phosphorus present in plasma and cells, samples were simply dried and read with the Geiger-Mueller tube and autoscaler. The size of the samples was dependent on the amount of  $P^{32}$  originally added to the blood. The radioactivity of the total acid soluble fraction of both plasma and red blood corpuscles was measured in dried samples of the trichloroacetic acid filtrates.<sup>8</sup> Inorganic phosphorus was determined by precipitation with calcium chloride as described by Fiske and Subbarow.<sup>8</sup> The precipitated calcium phosphate was redissolved in acid, and samples taken from this solution were dried in order to measure the concentration of radioactive phosphorus in the inorganic phosphorus fraction. Certain corrections for geometry, film thickness, and protein volume, were made and applied to all estimations of  $P^{32}$ .<sup>8</sup> It was estimated that the methods used were accurate to plus or minus 5 per cent to 10 per cent.

The results are expressed in one of several ways, either as the percentage of the amount of  $P^{32}$  added to the reaction flask, or as specific activities obtained by dividing the per cent of the added amount of  $P^{32}$  by the milligrams of the given phosphorus compound per 100 ml of plasma cells or whole bloods. The use of the percentage of added  $P^{32}$  rather than the number of counts as the basis of calculation permitted direct comparison of the different experiments despite variation in the actual amount of  $P^{32}$  added.

\*  $P^{32}$  was supplied by Monsanto Chemical Company, Clinton Laboratories, Oak Ridge, Tennessee.

† Tracerlab Boston, Massachusetts.

## RESULTS

*Distribution of Phosphorus Fractions between Plasma and Erythrocytes*

The data are presented in table 1. It will be observed that the distribution between red blood cells and plasma on the initial, nonincubated, blood were in the range of those normally accepted. After four hours incubation at 37.5 C (experiments 1, 2, 3) there was no net exchange between cells and plasma of any of the phosphate compounds beyond experimental errors of the methods employed. There was a slight increase of the plasma content of all of the forms of phosphorus studied as incubation proceeded. The hematocrit increased slightly or remained unchanged during the experiment. Minimal hemolysis was present in all samples.

TABLE 1—*Distribution of Phosphorus Compounds between Plasma and Erythrocytes*

Exp No	Tissue	Temp	Initial (5 minutes)				After 4 hours incubation			
			Hct *	Total P† mg per 100 ml	Inorganic P† mg per 100 ml	Total acid sol uble P mg per 100 ml	Hct	Total P† mg per 100 ml	Inorganic P† mg per 100 ml	Total acid sol uble P† mg per 100 ml
1	Plasma	37.5		12.8 (7.4)	3.0 (1.7)	3.1 (1.8)		14.7 (8.4)	4.4 (2.5)	4.7 (2.7)
	Red blood cells	37.5	41.8	63.4 (26.5)	7.3 (3.1)	38.8 (16.0)	43.0	51.2 (22.0)	7.8 (3.3)	34.6 (14.9)
2	Plasma	37.5		13.1 (7.4)	3.0 (1.7)	3.1 (1.7)		15.3 (8.7)	4.0 (2.3)	4.5 (2.5)
	Red blood cells	37.5	43.2	57.5 (24.8)	6.3 (2.7)	38.8 (16.8)	43.3	57.5 (25.1)	6.3 (2.8)	38.8 (16.5)
3	Plasma	37.5		11.6 (6.3)	2.9 (1.6)	2.8 (1.5)		15.2 (8.3)	5.0 (2.7)	5.6 (3.1)
	Red blood cells	37.5	45.3	55.2 (25.0)	7.3 (3.3)	31.4 (14.3)	45.5	47.6 (21.7)	7.7 (3.5)	33.2 (15.2)
4	Plasma	7		8.7 (5.0)	2.2 (1.3)	2.3 (1.3)		9.3 (5.4)	2.1 (1.2)	2.2 (1.3)
	Red blood cells	7	42.4	57.8 (24.5)	8.3 (3.5)	38.7 (16.4)	41.9	58.6 (24.6)	8.1 (3.4)	45.2 (18.9)

\* Hct—Hematocrit %

† Values given in mg per 100 ml of cells or plasma; figures in parentheses are values calculated for whole blood distribution from the hematocrit.

At 7 C, no change in hematocrit or distribution of phosphorus compounds occurred, nor was hemolysis of red blood cells observed.

There was no indication that inorganic phosphate of the red blood cells increased at the expense of the organic fraction during four hours of incubation at 37.5 C.

Acid soluble phosphorus in plasma was accounted for, within the limits of error, by the inorganic phosphorus both in the initial bloods and after the four hour incubation period at either 7 C or 37.5 C.

The data of table 1 include, in parentheses, a recalculation of the various phosphorus constituents as distributed in whole blood using the observed hematocrit and known dilutions of the blood as the basis of calculation.

When incubation was continued for twenty three hours at 37.5 C, a marked in-

crease of hematocrit was found and cell destruction, as indicated by marked hemolysis, was present. Concomitant with this cell destruction, marked increases in the plasma content of all the phosphorus fractions was found. For this reason data beyond an incubation period of four hours have been excluded from this communication.

### *Phosphate Exchange between Plasma and Erythrocyte*

The data are presented in table 2. It may be seen that between 91 and 105 per cent of the added  $P^{32}$  was recovered, or determined, as present in the plasma and red cells. These figures represent the limits of accuracy of the methods employed.<sup>5</sup>

TABLE 2 — *Phosphate Exchange between Plasma and Erythrocytes\**

Exp No	Tissue	Temp	Initial (5 minutes)				After 4 hours incubation		
			$P^{32}$ added† C.P.S. per 100 ml	Total P† % of added $P^{32}$	Inorganic P % of added $P^{32}$	Total acid soluble P % of added $P^{32}$	Total P† % of added $P^{32}$	Inorganic P % of added $P^{32}$	Total acid sol- uble P % of added $P^{32}$
1	Plasma Red blood cells	37.5	9 620	—	—	—	33.7 (4.0)	33.9 (13.6)	33.7 (12.5)
		37.5		—	—	—	57.2 (2.6)	43.6 (12.8)	57.4 (3.9)
2	Plasma Red blood cells	37.5	11 640	—	—	—	35.6 (4.1)	35.9 (15.6)	36.0 (14.4)
		37.5		—	—	—	51.6 (2.1)	46.0 (16.7)	52.2 (3.2)
3	Plasma Red blood cells	37.5	8 961	87.1 (13.8)	—	—	34.1 (4.1)	33.8 (12.5)	33.1 (10.7)
		37.5		3.6 (0.14)	—	—	55.7 (2.6)	49.8 (14.2)	57.8 (3.8)
4	Plasma Red blood cells	7	940	92.0 (18.5)	—	—	101.0 (18.7)	97.7 (81.4)	101.5 (78.1)
		7		6.7 (0.27)	—	—	2.9 (0.12)	2.6 (0.76)	3.2 (0.17)

\* All values calculated on whole blood distribution data

† C.P.S.—counts per second per 100 ml whole blood

<sup>5</sup> Figures in parentheses are specific activities calculated as  $\frac{\% \text{ of added activity per 100 ml}}{\text{mg of P compound per 100 ml}}$

In two experiments the per cent of the added  $P^{32}$  in the total phosphorus and specific activities were determined for plasma and red cells five minutes after the addition of the  $P^{32}$ . At this time 3.6 and 6.7 per cent were found in the red blood cells.

After incubation at 37.5 C for four hours, about one third of the added radio phosphorus was detected in the plasma. In each instance in the plasma there had been no turnover between the inorganic phosphate and the residual acid soluble organic or other organic forms. When blood was kept at 7 C for four hours all of the added  $P^{32}$  also remained in the plasma in the inorganic fraction in which form it had been added.

At 37.5 C the exchange of phosphate between plasma and red blood cells was rapid, from 51 to 57 per cent of the added radioactivity being detected in the washed red cells at four hours.

Most of the  $P^{32}$  was in the inorganic fraction of the acid soluble phosphate. From 6 to 14 per cent of the added  $P^{32}$  was found in the organic fraction of the acid soluble phosphorus. There was no indication that in four hours there was any exchange to the nonacid soluble organic fractions. At 7 C. essentially all of the  $P^{32}$  was in the inorganic form.

From a study of the specific activities presented in table 2, it will be seen that the specific activities of the inorganic phosphate of the cells equals or exceeds that of the inorganic fraction in the plasma at the end of four hours.

The ratio of the  $P^{32}$  activity, in terms of percentage of the isotope added, between the red blood cell and plasma  $\left( \frac{\text{per cent added } P^{32} \text{ in R B C}}{\text{per cent added } P^{32} \text{ in plasma}} \right)$  ranged between 1.4 and 1.7 with a mean of 1.5. In *in vivo* studies on five normal men five hours after injection of 100-200 microcuries of  $P^{32}$  this ratio was 6.4. In these instances the plasma level of  $P^{32}$  was rapidly falling for obvious physiologic reasons which were not duplicated in the *in vitro* experiments.

### DISCUSSION

The data presented amply confirm the statement of Eisenmann and her co-workers<sup>6</sup> that phosphate readily enters the red blood cells within a period of four hours at 37.5 C. The present investigations are free from any criticism that the entrance of the marked phosphate was due to the increased phosphate concentration of the plasma. Based, as they are, on direct determinations of  $P^{32}$  in both plasma and washed red cells, they show that the red blood cell is permeable to the phosphate ion. It is unfortunate that longer incubation studies were precluded by the physiologic changes in the red blood cell which culminated in its destruction by hemolysis. In the text it was indicated that incubation studies had been carried on beyond four hours at 37.5 C. Most of the data so obtained were vitiated by the hemolysis of the red cell. It is worth while mentioning that at twenty-three hours, the intracellular distribution of phosphorus compounds in the remaining cells did not differ markedly from those obtained at four hours. The slight increase in the plasma concentration of all the phosphorus derivatives is presumably due to slight hemolysis of the red blood cells.

The data indicate that while over 50 per cent of the added  $P^{32}$  entered the red blood cell at 37.5 C. in four hours, less than 15 per cent was found in the acid soluble organic fraction, the remainder having been present in the inorganic fraction. At this time the specific radioactivity of the red blood cell inorganic fraction was equal to or greater than the specific activity of the plasma inorganic fraction. Therefore, penetration and retention of phosphate in the red blood cell under these experimental conditions cannot be entirely accounted for by the formation of organic complexes.

It is well known that when whole blood is incubated at 37.5 C., the blood glucose rapidly falls, while when plasma or serum are so incubated the fall of glucose is very slow. It is possible that the transfer of phosphate from the inorganic to the organic acid soluble form may be due to phosphorylation which precedes the utilization of glucose by cells. This point is being further investigated.

Additional evidence that both the penetration of the red blood cell by phosphate and the turnover from inorganic to acid soluble organic phosphorus compounds may be functions of cell metabolism is given by a comparison of the data obtained at 37.5°C with that obtained at 7°C. In the latter case no significant exchange of labeled phosphate between red blood cells and plasma occurred and the small amount of  $P^{32}$  discovered in the red blood cell was confined entirely to the inorganic fraction.

This experience gives rise to the hope that *in vivo* studies of phosphate exchange in man can be undertaken using the red blood cell as a tissue cell model. If this is so, it will permit investigations of intermediate phosphate metabolism in man, where tissues such as the liver are not readily available.

#### SUMMARY

- 1 Phosphate exchange in red cells and plasma was studied *in vitro* using  $P^{32}$  in the form of sodium phosphate as a tracer.
- 2 No phosphate was added other than the isotopic preparation which was of high specific activity.
- 3 Inorganic phosphate exchanged freely between the plasma and the erythrocytes at 37.5°C in a period of four hours. Minimal transfer occurred at 7°C.
- 4 Most of the added  $P^{32}$  which passed into the erythrocytes during this time remained in the inorganic fraction, less than 15 per cent being found in the organic acid soluble fraction.
- 5 The specific activity of the inorganic phosphate of the erythrocytes was equal to or greater than that obtaining for the inorganic phosphate of the plasma at the end of the four hour incubation period at 37.5°C.

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